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VOLUME 46 JANUARY, 1950 NUMBER 1

ATTEMPTS TO PRODUCE DIABETES IN GUINEA PIGS BY ALLOXAN AND PANCREATECTOMY

With Observations on the Effect of a Diet Deficient in Cystine and Methionine

JEAN COLLINS-WILLIAMS, ALBERT E. RENOLD[1] AND ALEXANDER MARBLE

From the George F. Baker Clinic, New England Deaconess Hospital

BOSTON, MASSACHUSETTS

ALTHOUGH permanent diabetes has been produced in different ways in various species, it has not been reported in guinea pigs. Goldner (1945) observed degenerative lesions of the pancreas in guinea pigs only when they were given enough alloxan to cause death within 24 hours. Stoll (1946) reported that alloxan affected guinea pig islets but gave no details and Woerner (1947) noted damage to islets after 200 mg. per kg. An intense hypoglycemia persisting several days before death was observed by Saviano and DeFranciscis (1947) in guinea pigs injected intracardially with 50 to 450 mg. per kg. of alloxan. West and Highet (1948) found that 350 mg. per kg. intracardially caused death, but large doses were tolerated subcutaneously or intraperitoneally without diabetic or other symptoms. Charalampous and Hegstedt (1949) have confirmed their findings.

We have attempted subtotal pancreatectomy in young guinea pigs in a manner similar to that used by Shapiro and Pincus (1936) in young rats, but the blood vessels seemed more fragile than in rats and it was technically difficult to remove a large part of the pancreas while maintaining the circulation to the stomach and duodenum.

This paper describes some attempts to produce diabetes in guinea pigs with alloxan, and the effects of a diet deficient in cystine and methionine on the fasting blood sugar and the response to alloxan.

Received for publication June 27, 1949.

[1] Holder of a fellowship of the Swiss-American Center of Medical Exchange.

This work was aided by grants from the American Cyanamid Co. and the Diabetic Fund.

TABLE 1. EFFECTS OF INTRAVENOUS ADMINISTRATION OF ALLOXAN IN GUINEA PIGS

Guinea pig no.	Alloxan i.v. mg./kg.	Weight of animal gm.	Blood sugar, mg./100 cc.									Hemoglobin, gm./100 cc.		Notes
			Control 0 hr.	Highest noted 0–3 hr.	Lowest noted 2–9 hr.	Days after alloxan						Control 0 hr.	Lowest 1–6 days	
						1	2	3	4	5	6			
1	250	740	128	155	33	126	81	dead				14.0	3.1	
2	180	825	124	124	59	160	126	106	dead			13.3	0.7	NPN 145 mg./100 cc. on day 4.
3	180	545	95	130	46	dead						—	—	Died, accidental hemorrhage, 2 hr. after alloxan.
4	180	610	116	139	52	177	234	154	134	dead		12.2	1.0	NPN 124 mg./100 cc. on day 2.
5	180	695	109	109	42	dead						—	—	Died, accidental hemorrhage, 4 hr. after alloxan.
6	180	615	131	131	65	127	184	154	dead			—	—	
7	180	620	131	131	32	dead						—	—	
8	180	790	120	120	22	low	dead					—	—	
9	180	710	109	109	43	115	143	—	dead			12.8	6.2	NPN 238 mg./100 cc. on day 4.
10	160	590	122	122	99	119	155	124	154	151	dead	13.5	2.4	NPN 162 mg./100 cc. on day 5. Pretreated with tocopherol.
11	140	580	133	148	114	133	128	—	—	124	—	12.2	9.0	Survived.
12	140	520	130	153	106	135	139	—	—	—	—	13.3	10.0	Survived.
13	250	260	122	128	50	151	dead					11.3	8.7	
14	200	230	147	174	66	177	155	135	126	—	139	11.3	5.3	Survived.
15	200	215	101	156	58	148	141	116	130	—	130	11.5	6.1	Survived.
16	180	230	128	148	44	177	174	135	126	—	139	12.0	2.5	Survived.
17	180	230	114	144	78	157	122	—	139	126	—	13.4	7.0	Survived.
18	180*	600	142	270	135	143	181	130	190	148	145	10.7	9.0	Survived.
19	180*	605	—	242	94	129	166	dead				13.5	1.4	
20	180*	505	99	165	79	127	245	—	266	233	135	13.3	1.7	Survived.
21	180*	460	122	212	99	165	130	139	—	—	101	13.0	7.2	Survived.
22	180*	560	116	146	96	135	129	155	—	—	109	13.0	5.6	Survived.

* Given in 3 parts, 60 mg./kg. each, at 5 minute intervals.

GENERAL METHODS

Male guinea pigs weighing 500 to 800 gm. were used unless otherwise indicated. Alloxan (Eastman Kodak) was given as a 5 per cent solution in physiological saline intraperitoneally or intravenously. The ear veins were usually used, but for daily injections they could be used only about 4 times. Thereafter the superficial veins of the abdominal wall, the femoral or saphenous veins were exposed under local anesthesia and used for injections. Blood samples were usually obtained by nicking the ear veins.

Blood sugar was determined by the micromethod of Folin and Malmros (1929) and blood reduced glutathione by the Potter and Franke (1935) modification of the Benedict and Gottschall (1932) method, adapted to the Leitz photoelectric colorimeter.

For histological studies a part of the pancreas was fixed in Bouin's fluid and the remainder of the pancreas and portions of other organs in Helly's fluid. Gomori's (1941) chrome alum hematoxylin stain was used to differentiate beta and alpha cells in the pancreatic islets and hematoxylin and eosin stain was used on other tissues.

EXPERIMENTS AND RESULTS

Intraperitoneal Administration of Alloxan to Guinea Pigs. Single doses of alloxan up to 450 mg. per kg. were well tolerated intraperitoneally. Initial hyperglycemia at about 1 hour was noted but no subsequent hypoglycemia or diabetes occurred. Repeated doses of as much as 450 mg. per kg. each, totaling 725 mg. per kg. in 7 days, were given but only 1 animal had a blood sugar over 200 mg. per 100 cc. on 2 consecutive days.

Since Bruckmann and Wertheimer (1947) had reported that ammonium chloride given intraperitoneally in 5 per cent solution in dosage of 100 mg. per kg. increased the sensitivity of rats to alloxan, we used the same procedure with guinea pigs but observed no change in sensitivity. With 3 such doses in the 24 hours preceding alloxan, 2 animals died of undetermined causes at 5 and 7 hours after alloxan and 3 showed no change in sensitivity.

Intravenous Administration of Alloxan to Guinea Pigs. The doses used and results obtained with alloxan given intravenously are shown in Table 1. Adult guinea pigs injected with 180 mg. per kg. or more in a single dose developed hypoglycemia within 2 to 7 hours, frequently with convulsions. There was little or no initial hyperglycemia such as that after intraperitoneal administration of alloxan. Occasionally transiently elevated blood sugar levels occurred at 24 hours or later, the highest value recorded being 234 mg. per 100 cc. The blood nonprotein nitrogen was elevated and the hemoglobin markedly reduced. None of these animals lived longer than 5 days. Histological examination of the pancreas revealed degenerative lesions of the beta cells of the islets. One animal, dying of accidental hemorrhage during a hypoglycemic convulsion 2 hours after

the alloxan injection, already showed definite pyknosis and degranulation of beta cells, and most animals which died later had advanced necrosis of the islets. In the kidneys there was focal necrosis of the convoluted tubules, and frequently abundant hemoglobin casts in these and in the collecting tubules (Fig. 1). The picture resembled that in the human kidney following incompatible blood transfusions

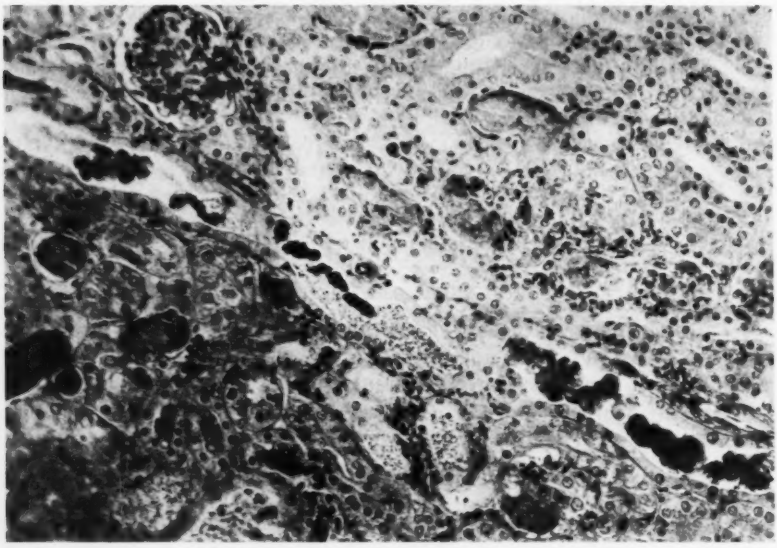

FIG. 1. Kidney of guinea pig injected intravenously with 180 mg./kg. of alloxan. An essentially normal glomerulus is seen in the upper left. Necrotic convoluted tubules are present (a group of three to right of center), and the long tubule seen coursing diagonally across the field contains pigment casts (brown in original preparation). Hematoxylin and eosin. ×250.

and is presumed to be a result of massive hemolysis. Extensive necrosis of the liver was observed in several of these animals.

One guinea pig given 160 mg. per kg. of alloxan died at 6 days with an elevated blood non-protein nitrogen and a low hemoglobin. The latter was not prevented by pretreatment with alpha-tocopherol, 30 mg. in oil, orally each day for the 6 days prior to alloxan administration. The use of tocopherol was suggested by the experience of Gyorgy and Rose (1948) who found that tocopherol-rich diets would prevent a decreased hemoglobin in rats after alloxan.

Young guinea pigs aged 5 to 6 weeks, given 180 to 200 mg. per kg. of alloxan, showed greater initial hyperglycemia and less, but definite, hypoglycemia than mature animals. They survived with only slight transient hyperglycemia after 24 hours (Fig. 2). Young guinea pigs to appear to be more resistant to the toxic effects of alloxan

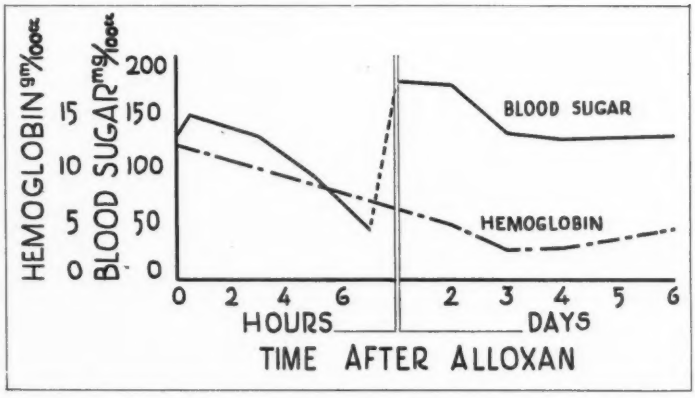

FIG. 2. A typical blood sugar and hemoglobin response in a young guinea pig after alloxan, 180 mg. per kg., intravenously. The animal survived with no apparent abnormalities.

than adults as in other species (Gitter and Prieto Diaz, 1946; Schultz and Duke, 1948).

When adult guinea pigs were given 180 mg. per kg. alloxan intravenously in 3 parts at 5 minute intervals the initial hyperglycemia was considerable and there was no hypoglycemia (Fig. 3).

Daily Intravenous Administration of Alloxan to Guinea Pigs. Three aspects of the action of alloxan in guinea pigs were then appar-

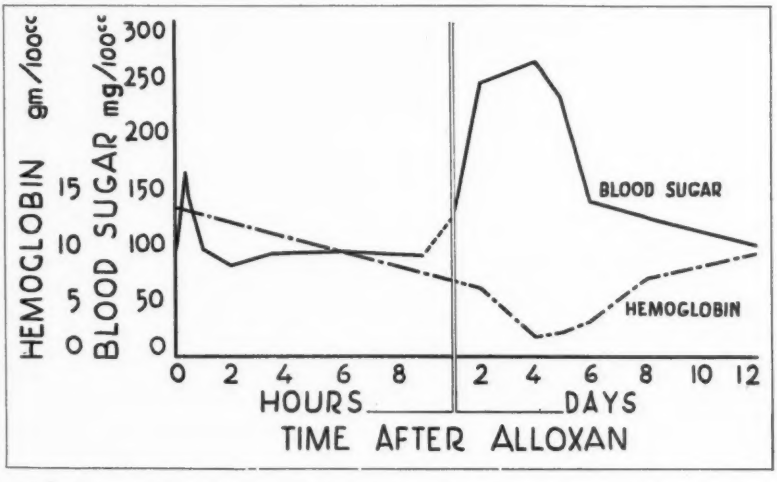

FIG. 3. A typical blood sugar and hemoglobin response in an adult guinea pig after alloxan, 180 mg. per kg. in 3 parts at 5 minute intervals, intravenously. The animal survived with no apparent abnormalities.

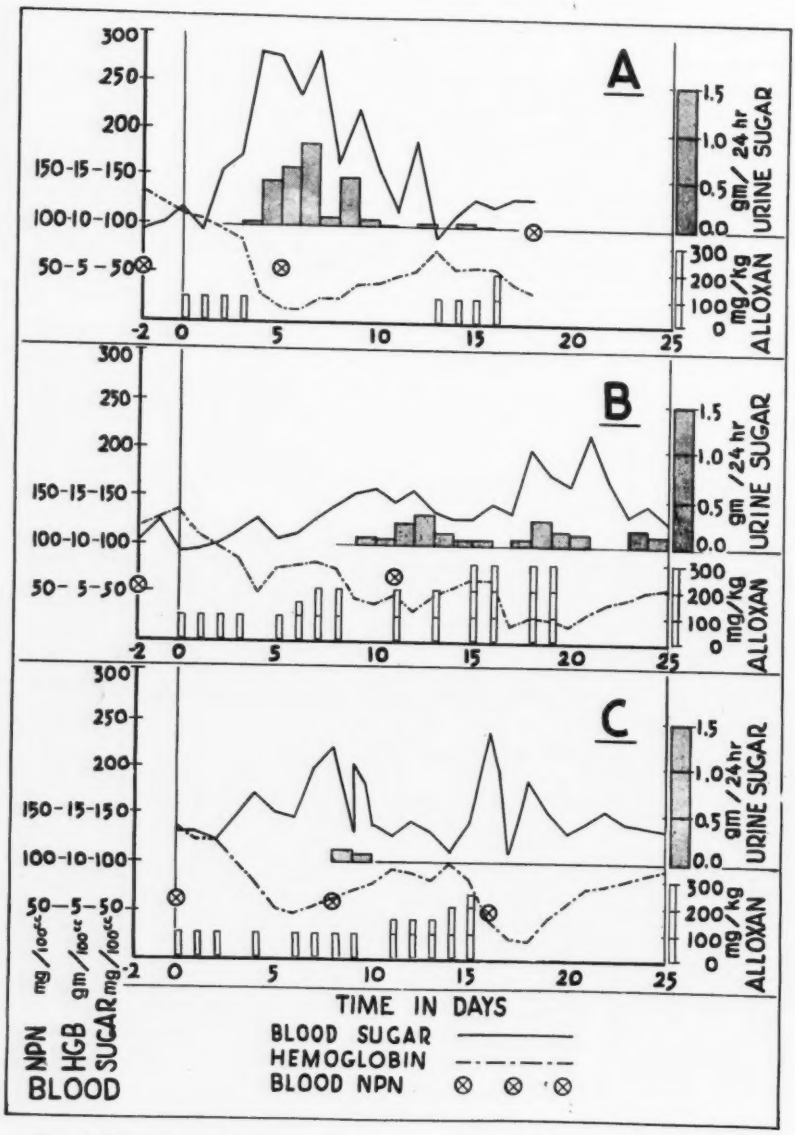

FIG. 4. The response to repeated intravenous injections of alloxan in 3 guinea pigs. (A and B, blood sugar determinations after overnight fast. C, random blood sugar determinations.)

ent: (1) the diabetogenic effect, (2) the hemolytic effect as evidenced by the precipitous fall in hemoglobin and the appearance of hemoglobin casts in the kidney tubules and (3) the toxic and frequently fatal effect on the kidney evidenced by azotemia and tubular necrosis. In a further attempt to separate the action on the pancreas from the other two effects, repeated intravenous injections of alloxan were given to 8 guinea pigs, usually at daily intervals, beginnning with doses of 100 mg. per kg. each. The daily hemoglobin determinations as well as the blood sugar level were used as indications of the effect of each dose and the injection was omitted or increased according to the rate of fall or rise in the hemoglobin.

Some animals died with the usual toxic symptoms and signs, including an elevated blood non-protein nitrogen and necrosis of the kidney tubules and pancreatic islets after as few as 2 daily injections of 100 mg. per kg. each. Others survived as many as 14 injections in 20 days, the last 4 of 300 mg. per kg. each. Hyperglycemia with considerable glycosuria was observed in 5 animals, 3 of which had fasting blood sugar values above 200 mg. per 100 cc. (Fig. 4). In 1 of these (Fig. 4A) the fasting blood sugar was above 280 mg. after only 4 injections of alloxan of 100 mg. per kg. each. After an interval of 9

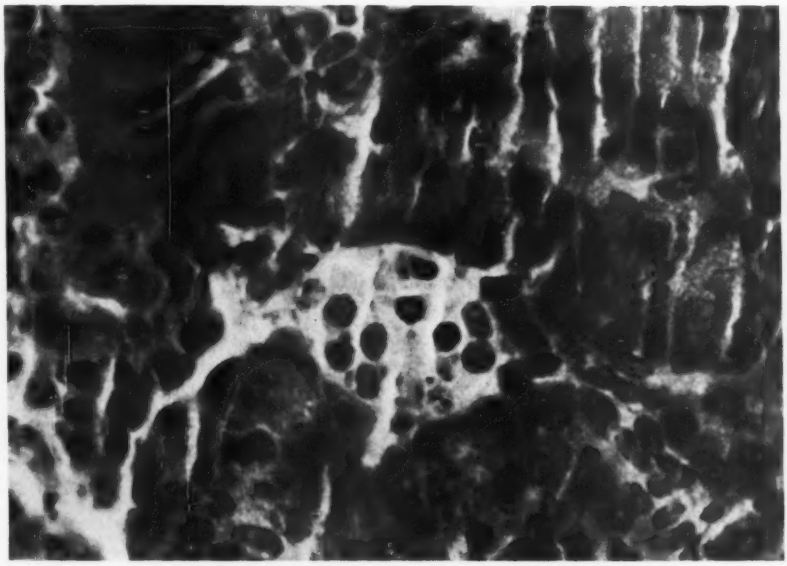

FIG. 5. Pancreas of guinea pig given serial injections of alloxan intravenously (see Fig. 4A). An islet, greatly reduced in size, is seen in the center of the field. Only a few such islets were found in several sections, and, although they appeared to consist chiefly of beta cells, it was evident that a considerable reduction in total mass of beta cells had occurred. Chrome-alum hematoxylin and phloxin. ×1000.

days, 4 more injections were given but due to the technical difficulties of giving more intravenous injections this animal was sacrificed, at which time the blood sugar was normal. Histologically the pancreatic islets seemed quite scarce and shrunken (Fig. 5) and there was minimal necrosis of the kidney tubules. The second course of alloxan injections in this animal probably accounts for a late increase in blood non-protein nitrogen and the kidney lesions, but the islet changes were of the type to be expected some time after alloxan and may be presumed to be due to the first course of injections.

One guinea pig (Fig. 4B) had blood sugar levels above 200 mg. only after 14 doses of alloxan, totaling 2650 mg. per kg. Biopsy of the pancreas at this time showed no acute necrosis of the islets but there appeared to be a marked reduction in the number of beta cells. In another guinea pig (Fig. 4C), as the hyperglycemia was decreasing

TABLE 2. BLOOD REDUCED GLUTATHIONE CONCENTRATION IN NORMAL ANIMALS AND MAN

Group	Number in group	Average hemoglobin concentration gm./100 cc.	Average blood reduced glutathione concentration mg./100 cc.
Rabbits (adults)	24	10.0±0.1*	43.8±0.3*
Rabbits (young)	12	9.1±0.1	38.6±0.4
Rats	5	13.9±0.1	47.8±0.5
Guinea pigs	17	13.9±0.3	59.3±0.8
Humans	6	13.7±0.3	35.3±1.0

* Standard error.

and the hemoglobin increasing with daily doses of 100 mg. per kg. a second hyperglycemic and anemic phase was produced by increasing the daily dose of alloxan. The pancreas of this animal appeared normal at necropsy.

Blood Glutathione Concentration in Relation to Alloxan Effects in the Guinea Pig. Leech and Bailey (1945) have shown that blood reduced glutathione levels fall rapidly after intravenous administration of alloxan in rabbits. Lazarow (1946) found that large quantities of glutathione or cysteine given to rats just before the alloxan prevented the diabetogenic effect. As guinea pigs are resistant to the diabetogenic action of alloxan it was considered that studies of the blood reduced glutathione might be of interest.

The blood reduced glutathione of guinea pigs was significantly higher than that of rabbits, rats and humans (Table 2). The average in 17 guinea pigs was 59 mg. per 100 cc. Decreases of 55 to 87 per cent occurred after injection of alloxan intravenously (Table 3).

To determine whether guinea pigs could be made more sensitive to the diabetogenic action of alloxan if the blood glutathione were decreased, Griffiths' modification (1948a) of the Haag and Wright

TABLE 3. THE EFFECT OF INTRAVENOUS ADMINISTRATION OF ALLOXAN ON THE BLOOD REDUCED GLUTATHIONE IN GUINEA PIGS

Guinea pig no.	Alloxan i.v. mg./kg.	Blood reduced glutathione mg./100 cc.		Decrease	
		control	2–12 min. after alloxan	mg./100 cc.	Per cent
1	250	49	20	29	55
2	180	60	8	52	87
3	180	60	19	41	68
4	180	57	15	42	74

(1940) cystine and methionine deficient diet was fed. Griffiths reported that on this diet or one containing less protein the blood glutathione in rabbits was lowered. The diet used for these guinea pigs contained 10 per cent of peanut meal, the sulfhydryl-deficient protein source.

All but 4 of 18 animals died in 1 to 4 weeks. By chance it was observed that 4 hours after its food was withdrawn one of the surviving guinea pigs had severe convulsions which were alleviated by glucose and artificial respiration. Subsequently in the same animal,

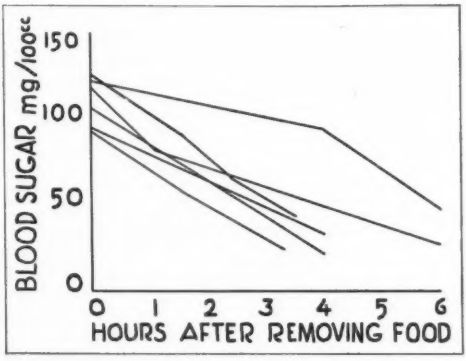

FIG. 6. Hypoglycemic response to withdrawal of food from guinea pigs after 3 to 6 weeks of a cystine and methionine deficient diet.

the blood sugar was determined at intervals after withdrawing food and a linear-type fall from 125 to 44 mg. per 100 cc. in 4 hours was observed.

It was apparent that before giving alloxan to any animal on the deficient diet the blood sugar response to fasting alone would have to be determined. In 6 of 10 guinea pigs after 3 to 6 weeks on the diet, severe hypoglycemia developed within 6 hours of fasting although the blood sugar had been normal at the time the food was withdrawn (Fig. 6). Hypoglycemia developed in 2 others even with food available.

When tissues of 5 animals which developed hypoglycemia on

fasting were examined histologically, the liver appeared normal in 2 and moderately fatty in 1. In the other 2 some shrinkage and vacuolation of cells around the central vein were seen. The adrenal glands appeared to be affected in 3 of the animals. Small foci of necrosis were observed in 1 and an extraordinary number of mitotic figures in the outer zona fasciculata in the other 2. The liver changes are apparently of a minor nature and the adrenal changes may be a nonspecific response to stress in the manner of Selye's general adaptation syndrome (1947). No other important histological changes were observed.

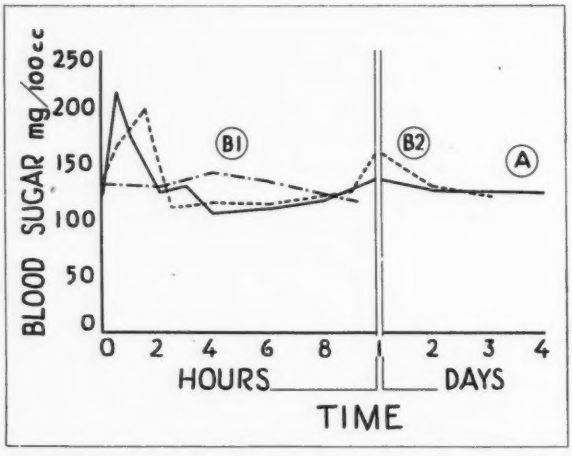

FIG. 7. Response of blood sugar in: (A) Control guinea pig, standard laboratory diet, 350 mg./kg. alloxan intraperitoneally. (B) Guinea pig after 4 weeks of sulfhydryl-deficient diet. Blood glutathione had fallen to 27 mg./100 cc. without development of hypoglycemic response to fasting. (1) Fasting alone. (2) Alloxan, 350 mg./kg. intraperitoneally. The similarity to control (A) is apparent.

This hypoglycemia during fasting was a chance finding and, as records of food intake and paired-fed controls are not available, it cannot definitely be attributed to lack of dietary cystine and methionine. However, as the diet was fed *ad libitum* and the animals ate it readily, it seems probable that the hypoglycemia resulted from the protein deficiency, rather than from a generally low food intake.

Only 2 guinea pigs on the deficient diet had a lowered blood glutathione at a time when the blood sugar remained normal for a 6 hour test period after withdrawal of food. When these 2 animals were given 350 mg. per kg. of alloxan intraperitoneally the blood sugar response was similar to that of normal guinea pigs (Fig. 7).

DISCUSSION

In common with other investigators we have found the guinea pig resistant to the diabetogenic action of alloxan. When alloxan was

administered *intraperitoneally*, large doses were tolerated. We have observed a consistent initial hyperglycemia within the first 2 hours not previously reported. In this respect the blood sugar pattern resembles that of rats during the first few hours after alloxan. However in the guinea pigs diabetes was not observed and there was only one transient hyperglycemic period lasting for 2 days.

After the administration of alloxan *intravenously* to such animals as the rabbit and dog, the blood sugar response is usually triphasic consisting of initial hyperglycemia within the first 2 hours, followed

TABLE 4. SUMMARY OF EXPERIMENTAL FINDINGS

Group	Alloxan mg. per kg.	Initial hyperglycemia (0–2 hr.)	Hypoglycemia (2–9 hr.)	Hyperglycemia (24 hr. or later)	Outcome
Adult guinea pigs	350–450 intraperitoneally	marked	absent	slight, transient (only 1 above 200 mg. per 100 cc.)	survived
Adult guinea pigs	180–250 intravenously	absent or slight	marked, frequent convulsions	slight, transient	died in 1–5 days
Adult guinea pigs	140–160 intravenously	absent	absent	absent	160—died 140—survived
Adult guinea pigs	180 in 3 parts at 5 min. intervals intravenously	marked	absent	occasional, transient (one 230 mg. for 4 days)	usually survived
Adult guinea pigs	100–300 per day for several days intravenously	not looked for	not looked for	*temporary diabetes*	variable
Young guinea pigs	180–250 intravenously	slight	moderate	occasional, transient (170–180 mg. per 100 cc.)	250—died 180–200 survived
Adult guinea pigs after cystine and methionine deficient diet	I. fasting only, no alloxan	—	marked	—	symptoms alleviated by sugar
	II. a. fasting only b. same animals 350 intraperitoneally	— marked	absent absent	— absent	survived

by profound hypoglycemia within 7 hours, and finally hyperglycemia again within 24 to 48 hours. In our experience all phases of this blood sugar pattern may be observed in guinea pigs after the intravenous injection of alloxan but all 3 phases are not usually seen in the same animal. The predominance of one or other phase seems to depend on the size and duration of the alloxan injection (Table 4). The hypoglycemic phase is predominant when a single large intravenous dose is given. Other investigators have not reported such a hypoglycemic phase after intracardiac or intravenous injection of alloxan in guinea pigs. A suggested explanation of the difference is that the injection may have been given more slowly in their anesthetized animals than in our unanesthetized animals in which the injection was rapid to avoid undue holding. The initial hyperglycemia is greater and the hypoglycemia absent if the alloxan is given in divided doses at 5 minute intervals. The final hyperglycemic phase is best obtained by repeated daily injections. Although this phase usually is permanent

in other animals, in the guinea pig the longest period of elevation of the blood sugar level over the control period accompanied by glycosuria has been 14 days.

This resistance of the guinea pig to the diabetogenic action of alloxan led to the studies of their blood reduced glutathione concentration. It was found to be higher than in other animals studied. In a recent report, Griffiths (1948b) also has observed that the blood reduced glutathione in guinea pigs is significantly higher than in rabbits. In view of the large amounts of glutathione which Lazarow had to inject to protect rats against alloxan, the higher blood glutathione in guinea pigs does not appear to be sufficient to account for the resistance It may, however, be a reflection of a higher glutathione in the tissues, especially in the pancreas.

In the experiments in which an attempt was made to lower the blood glutathione before giving alloxan the hypoglycemia observed during fasting alone necessitated great care in interpreting the effect of alloxan on the blood sugar.

While these experiments were in progress Griffiths (1948b) reported that, when the blood glutathione of 3 guinea pigs was lowered by a deficient diet to a level comparable with that of normal rabbits, the intraperitoneal injection of 200 mg. per kg. of alloxan was followed by marked hypoglycemia within 4 hours. Griffiths does not indicate the effect of fasting alone, without alloxan in such animals. In our experiments the only two guinea pigs showing a lowered blood glutathione without fasting hypoglycemia responded as normal animals to alloxan.

It may be concluded that by suitable serial intravenous injections of alloxan, transient diabetes can be produced in guinea pigs with hyperglycemia and glycosuria of several days' duration and with mild degenerative changes in the pancreatic islets. Although we have not been able to produce permanent diabetes either by pancreatectomy or by alloxan the results suggest that this may be due to the great difficulty of producing sufficient injury to the pancreatic islets without causing death from other toxic effects of alloxan on the blood and kidneys. The findings do not necessarily indicate any fundamental difference between the carbohydrate metabolism of guinea pigs and other animals.

SUMMARY

Guinea pigs developed temporary diabetes with hyperglycemia and glycosuria of several days' duration following the daily intravenous administration of alloxan in dosage of 100–300 mg. per kg. Permanent diabetes could not be produced because of the narrow margin between the diabetogenic dose and that which produced marked anemia and fatal kidney damage.

Guinea pigs tolerated large doses of alloxan intraperitoneally.

There was an initial hyperglycemic phase but no diabetes or kidney lesions. When 180 mg. per kg. was given intravenously marked hypoglycemia was observed within a few hours but no subsequent diabetes. The animals died within 5 days with severe hemolytic anemia, degenerative lesions of the pancreatic islets, and necrosis of the kidney tubules. When the same dose was given intravenously in 3 parts at 5 minute intervals, initial hyperglycemia was the predominant feature and the animals survived without hypoglycemia or diabetes.

Normal guinea pigs had an average blood reduced glutathione concentration of 59 mg. per 100 cc. which is higher than that of rabbits, rats or humans. It was decreased immediately after alloxan injection. After a diet deficient in cystine and methionine, severe hypoglycemia usually occurred within 6 hours after food was withdrawn. Only 2 animals had a lowered blood glutathione without this fasting hypoglycemia, and these responded as normal animals to the intraperitoneal injection of alloxan.

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THE EFFECT OF EPINEPHRINE UPON THE GLUCOSE TOLERANCE AND WORK PERFORMANCE OF THE EVISCERATE RAT

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IT WAS shown by Ingle and Nezamis (1949) that epinephrine can affect the glucose tolerance of the eviscerate rat and that the nature of the response is related to the experimental conditions. When glucose with insulin was given by constant intravenous injection to eviscerate rats, the addition of epinephrine caused a decrease in glucose tolerance within 2 hours which continued throughout 24 hours. These results were obtained in the inactive anesthetized animal in which the requirement for energy is low. The glucose tolerance of such animals is greatly increased by muscle work (Ingle and Nezamis, 1948) and it is well known that epinephrine has the property of increasing the work output of fatigued muscle. It was therefore proposed to study the effect of epinephrine upon the glucose tolerance and work performance of adrenalectomized and non-adrenalectomized eviscerate rats.

It was shown by this study that adrenalectomy causes a decrease in work output during the first 2 hours following operation associated with a small decrease in the level of blood glucose. The administration of epinephrine improved the work performance of the adrenalectomized-eviscerate rat without causing an increase in glucose requirement, thereby suggesting that in relationship to energy output epinephrine spares glucose utilization (oxidation, storage and conversion) in the working eviscerate rat just as in the resting eviscerate rat.

METHODS

Male rats of the Sprague-Dawley strain were fed Archer Dog Pellets. At a weight of 185 to 205 gm., the inferior vena cava was ligated between the liver and the kidneys in order to cause the development of a collateral circulation. Asepsis was preserved in this operation. When the animals reached a weight of 250 ± 2 gm., they were anesthetized (intraperitoneal injection of 18 mg. of cyclopentenyl-allyl-barbituric acid sodium) and eviscerated by the procedure of Ingle (1949). The stimulation of muscle was started immediately following evisceration. The procedure was according to Ingle (1944) with the following modifications. A Nerve Stimulator, Model B, Upjohn, was used to stimulate muscle at the rate of 5 times per second

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by a D.C. pulse of 20 milliamperes having a duration of 20 milliseconds. An electrode was placed on the lower tibia of the left back leg and the second electrode on the right back foot, thereby activating all of the musculature of both hind legs. The gastrocnemius muscle of the left hind leg was weighted with 100 grams. The distance that the weight was lifted was registered on automatic work recorders. Each recorder revolution represented approximately 400 gram-centimeters of work.

A solution of 0.9 per cent sodium chloride containing 1 mgm. of ascorbic acid per cc. and varying concentrations of glucose (C. P. Dextrose, Merck) and epinephrine hydrochloride (Upjohn) was infused into the jugular vein by means of a constant injection apparatus which delivered fluid from each of 12 syringes at the rate of 20 cc. in 24 hours. The ascorbic acid acted as an anti-oxidant which effectively preserved the epinephrine.

The animals were enclosed in a cabinet with temperature constant at $28 \pm 0.5°$ C. The infusions of glucose were started simultaneously with the stimulation of muscle immediately following the operation. The analyses of glucose were made on tail blood taken at the end of the 2-hour infusion period. The method was that of Miller and Van Slyke (1936).

TABLE 1. LEVELS OF BLOOD GLUCOSE IN ADRENALECTOMIZED AND NON-ADRENALECTOMIZED EVISCERATE RATS AT THE END OF 2 HOURS OF INFUSION WITHOUT WORK. MEANS AND STANDARD DEVIATIONS OF THE MEANS AND OF DIFFERENCES BETWEEN MEANS

Glucose load mgm./100/h.	Insulin units 24 hrs.	No. pairs rats	Eviscerate adrenal-ectomized	Eviscerate non-adrenal-ectomized	Difference
16	0	12	103 ± 8.9	115 ± 5.2	12 ± 10.3
18	0	20	115 ± 5.1	125 ± 4.6	10 ± 6.9
64	4	20	91 ± 5.2	107 ± 6.4	16 ± 8.2
72	4	13	139 ± 12.6	149 ± 20.3	10 ± 23.9

EXPERIMENTS AND RESULTS

The data on glucose loads, insulin dosage, epinephrine dosage, operation, numbers of animals and the average values for blood glucose at the end of 2 hours are summarized in Tables 1 through 6.

A comparison of the levels of blood glucose in eviscerate and adrenalectomized-eviscerate rats without work, in Experiment 1, (Table 1), showed that in each group the average level of blood glucose was lower in the adrenalectomized than in the non-adrenalectomized animals at the end of 2 hours of infusion, but that the differences were not sufficiently great to be considered reliable.

In Experiment 2 (Table 2) it was shown that the administration of epinephrine to eviscerate and adrenalectomized-eviscerate rats without work caused significant elevations of blood glucose in each group.

Experiment 3 (Table 3) was a study of the effect of epinephrine upon the glucose tolerance and work performance of non-adrenalectomized-eviscerate rats given glucose with and without insulin. Epinephrine in concentrations of 1:50,000 and less had little or no effect

TABLE 2. THE EFFECT OF EPINEPHRINE UPON THE TOLERANCE OF ADRENALECTOMIZED AND NON-ADRENALECTOMIZED RATS GIVEN GLUCOSE AND INSULIN INTRAVENOUSLY FOR 2 HOURS WITHOUT WORK. MEANS AND STANDARD DEVIATIONS OF THE MEANS AND OF DIFFERENCES BETWEEN MEANS

Operation	Glucose load mgm./100/h.	Epinephrine concentration	No. pairs rats	Blood glucose levels. Mgm. % at 2 hours		
				Epinephrine	No epinephrine	Difference
Adrenals out	68	1:50,000	12	231 ± 8.5	109 ± 13.1	122 ± 15.6
Adrenals out	64	1:50,000	12	234 ± 9.2	98 ± 3.6	136 ± 9.9
Adrenals out	64	1:100,000	20	195 ± 5.91	91 ± 5.17	104 ± 7.85
Adrenals intact	64	1:100,000	20	183 ± 6.13	107 ± 6.34	76 ± 8.82

TABLE 3. THE EFFECT OF EPINEPHRINE UPON THE GLUCOSE TOLERANCE AND WORK PERFORMANCE OF NON-ADRENALECTOMIZED-EVISCERATE RATS GIVEN GLUCOSE INTRAVENOUSLY FOR 2 HOURS. MEANS AND STANDARD DEVIATIONS OF THE MEANS

Glucose load mgm./100/h.	Insulin units per 24 hrs.	Epinephrine	No. pairs rats	Epinephrine			No epinephrine		
				Final rate	Total work	Final glucose	Final rate	Total work	Final glucose
160	0	1:100,000	12	29 ±3.6	4421 ±415	76 ±7.7	31 ±2.2	4519 ±270	79 ±12.2
160	0	1: 50,000	12	40 ±1.8	4774 ±217	95 ±9.4	38 ±2.4	5032 ±312	93 ±10.6
160		1: 25,000	6	30 ±4.8	4043 ±513	158 ±22.4	37 ±4.9	4527 ±592	108 ±20.5
220	4	1:100,000	12	35 ±2.4	4432 ±232	100 ±19.5	33 ±2.6	4363 ±329	93 ±14.7
220	4	1: 50,000	12	26 ±2.1	3875 ±343	111 ±25.8	31 ±2.9	4619 ±259	71 ±13.5
240	4	1: 50,000	12	33 ±2.6	4694 ±340	122 ±19.4	38 ±2.2	4752 ±247	103 ±10.9
240	4	1: 25,000	12	32 ±2.9	3339 ±328	262 ±33.8	38 ±3.3	4817 ±205	127 ±9.2

upon the work output or the glucose tolerance, but 1:25,000 epinephrine suppressed work performance and caused a marked rise in blood glucose.

Experiment 4 (Table 4) was a study of the effect of epinephrine upon the glucose tolerance and work performance of eviscerate and adrenalectomized-eviscerate rats given glucose within insulin. The adrenalectomized rats without epinephrine performed less work than non-adrenalectomized rats and the administration of epinephrine sustained work performance at a significantly higher level with a small increase in blood glucose. The administration of epinephrine to non-adrenalectomized-eviscerate rats did not improve work output.

When the results for 65 pairs of eviscerate and adrenalectomized-eviscerate rats given a glucose load of 240/100/h. with insulin are

TABLE 4. THE EFFECT OF EPINEPHRINE UPON THE GLUCOSE TOLERANCE AND WORK PERFORMANCE OF ADRENALECTOMIZED AND NON-ADRENALECTOMIZED EVISCERATE RATS GIVEN GLUCOSE (240/100/H.) AND INSULIN (4 UNITS PER 24 HRS. PER) RAT INTRAVENOUSLY FOR 2 HOURS. MEANS AND STANDARD DEVIATIONS OF THE MEANS

Operation	Epinephrine concentration	No. pairs rats	Epinephrine			No epinephrine		
			Final rate	Total work	Final glucose	Final rate	Total work	Final glucose
Adrenals intact	1:100,000	24	29 ±2.2	4491 ±164	76 ±5.2	31 ±2.0	4506 ±111	74 ±5.4
Adrenals out	1:100,000	24	28 ±2.2	4145 ±110	82 ±8.4	17 ±1.1	3349 ±108	71 ±4.2
Adrenals out	1:100,000	15	27 ±2.3	4429 ±303	93 ±10.4	19 ±1.1	3375 ±176	73 ±7.3
Adrenals out	1: 50,000	24	30 ±1.7	4352 ±187	88 ±6.3	22 ±1.1	3730 ±121	76 ±4.2
Adrenals intact	1: 50,000	24	30 ±2.2	3847 ±186	105 ±7.3	33 ±1.5	4542 ±162	84 ±7.1

TABLE 5. THE EFFECT OF ADRENALECTOMY UPON THE WORK PERFORMANCE AND GLUCOSE TOLERANCE OF EVISCERATED RATS GIVEN GLUCOSE (240/100/H.) AND INSULIN (4 UNITS PER 24 HOURS PER RAT) DURING 2 HOURS. POOLED RESULTS FOR 65 PAIRS OF RATS. MEANS AND STANDARD DEVIATIONS OF MEANS AND OF DIFFERENCES

	Eviscerate adrenalectomized	Eviscerate non-adrenalectomized	Differences in means
Final rate	19 ± 0.7	34 ± 1.1	15 ± 1.3
Total work	3495 ±87.7	4623 ±90.6	1128 ±126.1
Final glucose	73 ± 2.7	77 ± 4.1	4 ± 4.9

pooled together (Table 5), it is apparent that removal of the adrenal glands from the eviscerate rat caused a significant decrease in work performance and a slight, unreliable decrease in the level of blood glucose. When the results on adrenalectomized-eviscerate rats given a glucose load of 240/100/h. with insulin were pooled together (Table

TABLE 6. THE EFFECT OF EPINEPHRINE UPON THE GLUCOSE TOLERANCE AND WORK PERFORMANCE OF ADRENALECTOMIZED EVISCERATE RATS GIVEN GLUCOSE (240/100/H.) AND INSULIN (4 UNITS PER 24 HOURS PER RAT) DURING 2 HOURS. POOLED RESULTS. MEANS AND STANDARD DEVIATIONS OF THE MEANS AND OF DIFFERENCES

	1:100,000 epinephrine	No epinephrine	Differences in means
Number of rats	39	65	
Final rate	24 ± 1.6	19 ± 0.7	5 ± 1.7
Total work	4191 ±131.2	3495 ±87.7	696 ±158
Final glucose	86 ± 6.4	73 ± 2.7	13 ± 6.9

6), it was shown that the administration of 1:100,000 epinephrine caused a significant increase in the work output and a small rise in the amount of blood glucose which did not quite meet the usual requirements for statistical reliability.

DISCUSSION

Russell (1943), Roberts (1946) and Ingle and Nezamis (1948) have noted that adrenalectomy accelerates the rate at which glucose

disappears from the blood of the eviscerate rat. Ingle and Nezamis observed that the effect is established within 4 hours following adrenalectomy and recognized that adrenal medullary insufficiency may have been a factor in causing the increase in the glucose tolerance. When the animals have been studied 2 hours following removal of the adrenal glands, the level of blood glucose has always been lower in the adrenalectomized than in non-adrenalectomized animals (Tables 1, 2 and 4). Although the differences have been small and are of low statistical reliability, the direction of the difference has been consistent. It seems probable that the effect of adrenalectomy in causing a small increase in glucose tolerance has its beginning within the first 2 hours following operation.

The effect of epinephrine in suppressing the glucose tolerance of eviscerate rats given insulin might be explained by the hypothesis (Long, 1946) that epinephrine stimulates the release of ACTH by the anterior pituitary which activates the adrenal cortex to release increased amounts of its "diabetogenic" steroids. The presence of the adrenal cortices is not essential for the effect of epinephrine upon glucose tolerance as was demonstrated by the results of Experiment 2 (Table 2).

When epinephrine is given to the resting eviscerate rat in a concentration of 1:100,000 with glucose and insulin, it causes a significant rise in the level of blood glucose (Table 2), but the same concentration given to working eviscerate rats with glucose and insulin (Tables 3 and 4) had only slight, statistically unreliable effects upon the level of blood glucose. When given in higher concentrations, it suppressed the output of work in eviscerate rats given glucose with and without insulin and caused a rise in the level of blood glucose (Tables 3 and 4). Is work suppressed because of the inhibition in glucose utilization, or is there a decrease in glucose utilization because work output is limited by some other mechanism? We are not able to answer this question.

Adrenalectomy in the eviscerate rat (Table 5) causes a definite decrease in work output with an insignificant decrease in the level of blood glucose. These changes are reversed by the administration of epinephrine to the adrenalectomized-eviscerate animal (Table 6), although the administration of epinephrine to non-adrenalectomized eviscerate rats failed to improve work performance under these conditions. These results can be explained by the assumption that the adrenal medullae are stimulated to secrete optimal amounts of epinephrine under the conditions of these experiments. This would also explain the observation that the administration of 1:50,000 epinephrine increased the work output of the adrenalectomized-eviscerate rat but suppressed the work output of the non-adrenalectomized animals, since in the latter case the exogenous epinephrine

would be added to an already optimal endogenous level of epinephrine and the sum would represent an overdosage level.

Although epinephrine did not significantly depress glucose tolerance except in doses which suppressed work output, it did cause significant increases in work in the adrenalectomized animals without any increase in glucose requirement, and there was evidence for a small concomitant decrease in glucose requirement, thereby indicating that it spared the utilization of glucose in relation to energy output.

These observations upon the early effects of adrenalectomy upon work are consistent with the observations of Ingle and Lukens (1941) that adrenal demedullation suppresses work output during the early hours of muscle stimulation, although the adrenal demedullated animal can perform as much total work as normal animals (Ingle, Hales and Haslerud, 1936) and that the continuous administration of epinephrine does not modify total work in the adrenalectomized, non-eviscerated rat (Ingle and Nezamis, in press).

The changes in glucose tolerance and work performance which occur within a short time following adrenalectomy and their reversal with epinephrine provide indirect evidence that the secretion of the adrenal medulla during the stress of these procedures plays a physiological role in muscular responsiveness and carbohydrate metabolism which is, in part at least, by extra hepatic mechanisms.

SUMMARY

Male rats were eviscerated at a weight of 250 grams and were given continuous intravenous infusions of solutions containing glucose with and without insulin and with and without epinephrine for a period *of* 2 hours. The rate of infusion was 20 cc. per 24 hours. Adrenalectomy in the eviscerate rat caused a small increase in the tolerance for glucose within 2 hours. Epinephrine caused a decrease in glucose tolerance of eviscerate rats given insulin in both the presence and absence of the adrenal glands. Epinephrine failed to improve the work performance of eviscerate rats having the adrenal glands intact and had little effect upon the level of blood glucose at a concentration of 1:100,000 although this dose of epinephrine caused a striking decrease in glucose tolerance in the non-working, eviscerate rat. Larger doses of epinephrine (1:50,000 and 1:25,000) suppressed work output and caused a decrease in glucose tolerance. Adrenalectomy in the eviscerate rat caused a significant decrease in work output with an accompanying small increase in glucose tolerance. These changes were reversed by the administration of epinephrine (1:100,000 and 1:50,000) to the adrenalectomized-eviscerate rat. It was concluded that these data support the hypothesis that the physiological secretion of epinephrine by the adrenal medulla during stress

has an effect upon work performance during the early hours of muscle stimulation and modifies glucose tolerance, in part at least, by extra hepatic mechanisms.

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A COMPARATIVE STUDY OF SOME OF THE CHEMICAL AND MORPHOLOGICAL CHANGES ELICITED IN THE ADRENALS BY STRESS AND PURIFIED ACTH

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INTRODUCTION

ENLARGEMENT of the adrenal gland (Selye, 1936, 1937) with a discharge of its cholesterol (Popjack, 1944, Levin, 1945, Long, 1947, Ludewig and Chanutin, 1947), ascorbic acid (Giroud, 1940, Torrance, 1940, Sayers *et al.*, 1945) and histologically demonstrable lipids (Zwemer, 1936, Selye, 1936, 1938, Dalton, Dosne and Selye, 1940, Darrow and Sarason, 1944) are generally regarded as evidence of increased adrenal activity during stress. The prevention of these changes by hypophysectomy (Selye, 1937) and their reproduction with purified ACTH (Sayers *et al.*, 1946) has clearly established the rôle of this pituitary hormone in the regulation of adrenal-cortical function.

However, in previous experiments with repeated injections of a crude anterior-pituitary preparation (LAP) we failed to observe any discharge of histologically demonstrable cortical lipids and indeed inhibited the depletion of sudanophilic material normally produced by stilbestrol (Skelton *et al.*, 1949) or other non-specific stressing agents (Fortier *et al.*, 1949). The absence of adrenal stainable lipid depletion had also been observed after repeated injections of purified ACTH in some of our earlier investigations. In order to control the time-factor, it appeared necessary to compare the response of the adrenal in this respect following single and multiple doses of ACTH. Curiously, repeated injections of ACTH did not appear to reproduce the adrenal response to systemic stress; hence we also wanted to compare the chemical and morphological changes produced in the adrenal, on the one hand by sustained stress, and on the other, by repeated injections of ACTH given during the same period.

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EFFECT OF A SINGLE LARGE DOSE OF ACTH AT ONE, THREE AND TWELVE HOUR INTERVALS

Materials and methods.—To establish the pattern of the adrenal response to a single injection of ACTH, 36 male piebald rats averaging 150 gm. in weight were divided into three groups. Group I consisted of nine rats which were used as absolute controls. Group II was divided into three sub-groups of three animals each, which were respectively killed, one, three and twelve hours after receiving 12 mg. of a foreign protein (hypertensinogen). Group III was similarly divided into three sub-groups of six rats which were killed at the same intervals following the injection of 12 mg. of ACTH (Armour, Lot #58–9 (50)). Both hypertensinogen and ACTH were administered in 0.2 ml. of normal saline, subcutaneously. All animals were fasted for a uniform 24 hour period prior to killing. Immediately after the animals were killed by bleeding, both adrenals and thymus were removed. The right adrenal was fixed in Bouin's solution for frozen sections at 12μ and stained for fat with Sudan IV. The left adrenal was dissected free of fat and connective tissue, weighed fresh and ground in 4% trichloracetic acid for ascorbic acid determination (method of Roe and Kuether, 1943). The thymus was also fixed in Bouin's solution and weighed on an analytical balance.

Results.—The organ weights and adrenal ascorbic acid values for the different time intervals are summarized in Table 1. As can be seen, significant changes in adrenal weight were not observed at any time in any of the groups. On the other hand, the adrenal ascorbic acid, though uninfluenced by the foreign protein, showed a marked drop in the ACTH-treated animals. Maximal depletion occurred

TABLE 1. EFFECT OF A SINGLE LARGE DOSE OF ACTH AT ONE, THREE AND TWELVE HOUR INTERVALS ON ADRENAL WEIGHT AND ASCORBIC ACID CONCENTRATION*

		Hours after injection							
		0 hrs		1 hrs		3 hrs		12 hrs	
Group	Treatment	Weight	Ascorbic acid	Weight	Ascorbic acid	Weight	Ascorbic acid	Weight	Ascorbic acid
I	None	13.3 ±1.1	0.541 ± .071						
II	Foreign Protein† (Hypertensinogen) 12 mg			14.4 ±1.32	0.551 ± .029	14.6 ±0.3	0.501 ± .060	14.2 ±0.5	0.572 ± .033
III	ACTH† 12 mg.			12.6 ±0.88	0.274 ± .011	13.8 ±1.32	0.301 ± .014	13.6 ±1.08	0.523 ± .016

* The Adrenal Weight is expressed in mg./100 g. of Body Weight, the Ascorbic Acid in mg./100 mg. of Adrenal Tissue.
† Dissolved in 0.2 cc. of Physiological Saline and injected subcutaneously.

after one hour (49.3% below the initial level) and this was followed by a gradual increase to normal values, twelve hours after injection.

Microscopic examination showed a slight depletion of sudanophilic material in the reticularis and inner fasciculata of the hypertensinogen-injected controls, one and three hours after injection. In the ACTH-injected rats the reticular and inner fascicular zones of the adrenals were more markedly (though incompletely) depleted of

lipids one hour after injection. After three hours, the lipid discharge was intense and involved all three layers of the cortex, except a narrow band in the glomerulosa. However, twelve hours after ACTH administration, the lipid distribution had returned almost to normal; only the reticularis continued to show a slightly reduced staining intensity.

EFFECT OF REPEATED DOSES OF ACTH OVER A 24 HOUR PERIOD

Materials and methods.—In this experiment, 16 male, piebald rats, averaging 65 gm. in weight were divided into two groups. The animals of group I served as controls and each received six doses of 0.2 ml. of normal saline, subcutaneously, equally distributed over a 24 hour period. The rats of group II received a 20 mg. dose of Armour's purified ACTH (Lot #32 D) also distributed in six subcutaneous injections of 0.2 ml. during the same period. The animals were fasted for the duration of the experiment and then killed by exsanguination, one hour after the last injection. Both adrenals and thymus were immediately removed, fixed in Bouin's fixative and weighed. One adrenal was used for frozen sections at 12μ and stained for fat with Sudan IV.

Results.—The results summarized in Table 2 reveal that neither the saline nor the ACTH treatment had any effect on the adrenal

TABLE 2. EFFECT OF REPEATED DOSES OF ACTH OVER A 24 HOUR PERIOD* ON ADRENAL AND THYMUS WEIGHT

Group	Treatment	Adrenal weight		Thymus weight	
		mg./100 g.	P†	mg./100 g.	P
I	Saline inj.	16.0 ± 0.6		213 ± 11	
II	ACTH, 20 mg.‡	16.2 ± 1.0	II–I > 0.1	118 ± 8	II–I < 0.01

* The animals were killed one hour after the last injection.

† Probability of a chance occurrence of the difference between the means, from Fisher's Table of T values.

‡ Administered in six equally spaced subcutaneous injections of 3⅓ mg. dissolved in 0.2 cc. of physiological saline.

weight. ACTH had however a significant thymolytic effect in the experimental group (34.9% below the normal value). The sudan staining of the adrenal glands revealed an equal and slight depletion of lipids from the reticularis in both the saline and ACTH-treated rats.

COMPARISON OF CHANGES CAUSED BY REPEATED ACTH INJECTIONS AND BY SEVERE SYSTEMIC STRESS OVER A 48 HOUR PERIOD

Materials and Methods.—Twenty-four female piebald rats weighing an average of 65 gm. were used in this experiment. They were equally subdivided into the following four groups:

Group I served as absolute control. Group II received, over a 48 hour period, 24 equally spaced subcutaneous injections of 0.2 ml. of physiological saline. Group III received the total amount of 38 mg. Armour's purified

ACTH (Lot #32 D) according to the same procedure. In group IV, the spinal-cord was transected just caudad to the 7th cervical vertebra. The animals of groups I, II and III were allowed Purina Fox Chow and water *ad lib.*, while those of group IV were fasted throughout the experimental period, since starvation notoriously augments the adrenal response to systemic stress. All animals were killed by bleeding and immediately thereafter, the adrenals and thymus were removed and fixed as in the first experiments. Of one adrenal, frozen sections were prepared at 12μ and stained for lipid with Sudan IV. The other adrenal and the thymus were sectioned and stained with H and E.

Results.—Table 3, which contains a summary of the experimental findings, shows that both the absolute control and saline-injected animals (Groups I and II) gained weight during the 48 hour period, while the ACTH-treated and spinal-cord transected animals (Groups

TABLE 3. COMPARISON OF CHANGES CAUSED BY REPEATED ACTH INJECTIONS AND BY SEVERE SYSTEMIC STRESS OVER A 48 HOUR PERIOD ON ADRENAL AND THYMUS WEIGHT

Group	Treatment	Adrenal weight		Thymus weight	
		mg./100 g.	P*	mg./100 g.	P
I	None	19.4 ±1.3		369 ±27	
II	Saline inj.	19.5 ±1.2	II–I <0.1	389 ±35	II–I <0.1
III	ACTH 38 mg.†	45.3 ±1.6	III–I <0.01 III–II <0.01	88 ±8	III–I <0.01 III–II <0.01
IV	Spinal Cord Transection	36.2 ± 2	IV–I <0.01 IV–II <0.01 IV–III <0.01	173 ±10	IV–I <0.01 IV–II <0.01 IV–III <0.01

* Probability of a chance occurrence of the difference between the means, from Fisher's Table of T values.

† Administered in 24 equally spaced subcutaneous injections of 1.6 mg. dissolved in 0.2 cc. of physiological saline.

III and IV) lost weight. The adrenal and thymus weights of groups III and IV are markedly different from those of either groups I or II; the adrenals are significantly enlarged while the thymus is atrophic. It is noteworthy that ACTH, in the dose used, was more effective in producing adrenal hypertrophy and thymus involution than the systemic stress of spinal-cord transection.

Histologically, the adrenals and thymus showed the following: The adrenal-lipid pattern was normal in groups I and II (absolute and saline-injected controls), while in group III (stress) there was an almost complete depletion of cortical sudanophilic material. Group IV (ACTH), however, had a normal or even increased lipid content, which at high magnification, proved to result from a considerable increase in the number of sudanophilic granules.

The H and E sections of the thymus of groups I and II were nor-

mal while those of groups III and IV presented a picture of nuclear disintegration, hemorrhages, fusion of cortical and medullary structure, prominence of reticulum cells and macrophages, all of which is characteristic of increased adrenal cortical activity. The adrenals of the spinal-cord-transected and ACTH-treated animals showed the expected cortical hyperplasia and hyperemia.

COMPARISON OF CHANGES CAUSED BY REPEATED ACTH INJECTIONS AND BY SEVERE SYSTEMIC STRESS OVER A 24 HOUR PERIOD

Materials and Methods.—For this experiment, 72 male piebald rats, averaging 65 gm. in weight, were divided into eight groups as follows:

Group I served as absolute controls. Group II was injected subcutaneously with 0.24 ml. of physiological NaCl solution, at two hour intervals during 24 hours. Group III received the same injections as Group II, but in addition, the animals were fasted for a period of 48 hours, starting 24 hours before the beginning of the experiment. Group IV was injected every two hours for 24 hours, with 0.24 ml. of a solution containing 1.16 mg. of electrophoretically pure ACTH, kindly supplied by Dr. C. H. Li. In this way, each animal received a total of 14 mg. Group V received the same treatment as group IV but was, in addition, fasted during 48 hours as group III. Group VI received a total dose of 0.1 mg. of Li's ACTH solution distributed in two-hour injections of 0.24 ml. each (each single injection therefore consisting of approximately 0.083 mg.). Group VII was treated like Group VI, but was fasted during 48 hours as group III. Group VIII was submitted to fasting and cold (1° C.) simultaneously during the same period.

The animals of groups I, II, IV and VI were fed Purina Fox Chow *ad lib.* All groups were given tap water to drink.

The injections were so timed that each animal was sacrificed (by bleeding) exactly two hours after the last injection. Immediately after killing, both adrenals were weighed fresh. Their cholesterol and ascorbic acid contents were determined respectively by the methods of Sperry (1938) and Roe and Kuether (1943).

In addition to the above animals required for chemical determinations, it was possible to include in each group three animals for histological study. Both adrenals and thymus were fixed in Bouin's solution for 48 hours, then weighed and cut at 6μ for H and E and at 10μ (frozen sections) for Sudan staining.

Results.—A summary of the results is given in Table 4, which includes a statistical comparison of our values.

The injection of NaCl (Group II) causes no significant change from the normal (Group I) in the weight, cholesterol or ascorbic acid content of the adrenals. However, in group III, which was fasted and NaCl-injected there was a significant enlargement and a decrease in the cholesterol and ascorbic acid content of the adrenals. The apparent thymus involution in this group is not significant.

The large dose of ACTH (Group IV) caused a marked adrenal hypertrophy, thymus involution and a concomitant fall in adrenal cholesterol and ascorbic acid; all these values differ significantly

TABLE 4. COMPARISON OF CHANGES CAUSED BY REPEATED ACTH INJECTIONS AND BY SEVERE SYSTEMIC STRESS OVER A 24 HOUR PERIOD* ON THYMUS WEIGHT AND ADRENAL WEIGHT, CHOLESTEROL AND ASCORBIC ACID CONTENT

Group	Treatment	Thymus weight		Adrenal weight		Adrenal cholesterol		Adrenal ascorbic acid	
		mg./100 g. body	P†	mg./100 g. body	P	mg./100 mg. body	P	mg./100 mg. tissue	P
I	None	448 ±34		20.2 ±1.4		2.670 ±0.252		0.464 ±0.026	
II	Saline	430 ±48	II-I > .1§	21.9 ±1.6	II-I >0.1§	2.245 ±0.420	II-I§ >0.1	0.352 ±0.076	II-I >0.1§
III	Saline+fasting	348 ±39	III-I <0.1§ III-II >0.1§	32.4 ±2.5	III-I <0.01 III-II <0.01	1.472 ±0.121	III-I <0.01 III-II >0.1§	0.349 ±0.029	III-I <0.02 III-II >0.1*
IV	ACTH‡ 15 mg.	274 ±16	IV-I <0.01 IV-II <0.01	36.5 ±1.7	IV-I <0.01 IV-II <0.01	0.866 ±0.067	IV-I <0.01 IV-II <0.01	0.162 ±0.032	IV-I <0.01 IV-II <0.05
V	ACTH‡ 15 mg. +Fasting	258 ±19	V-I <0.01 V-III < .1§ V-IV >0.1§	41.2 ±3.1	V-I <0.01 V-III <0.05 V-IV >0.1§	0.717 ±0.049	V-I <0.01 V-III <0.01 V-IV >0.1§	0.130 ±0.049	V-I <0.01 V-III <0.01 V-IV >0.1§
VI	ACTH‡ 0.01 mg.	412 ±32	VI-I >0.1§ VI-II >0.1§	24.0 ±1.5	VI-I <0.02 VI-II >0.1§	2.319 ±0.330	VI-I >0.1§ VI-II >0.1§	0.478 ±0.022	VI-I >0.1§ VI-II <0.1§
VII	ACTH‡ 0.01 mg. +Fasting	309 ±39	VII-I <0.02 VII-III >0.1§ VII-VI <0.1§	38.4 ±1.7	VII-I <0.01 VII-III >0.1§ VII-VI <0.01	1.414 ±0.159	VII-I <0.01 VII-III >0.1§ VII-VII <0.05	0.454 ±0.013	VII-I >0.1§ VII-III <0.01 VII-VI >0.1§
VIII	Cold+Fasting	263 ±29	VII-I <0.01 VII-V >0.1§	42.5 ±2.9	VIII-I <0.01 VIII-V >0.1§	0.859 ±0.101	VIII-I <0.01 VIII-V >0.1§	0.377 ±0.044	VIII-I >0.1§ VIII-V <0.1

* The animals were killed two hours after the last injection.
† Probability of a chance occurrence of the difference between the means, from Fisher's Table of T Values.
‡ Dissolved in physiological saline, and subcutaneously injected every 2 hours for 24 hours.
§ Difference not statistically significant.

from those of both the normal (I) and the corresponding experimental control group (II). The seeming enhancement of these differences by fasting (Group V) is not significant. The small (0.1 mg.) dose of ACTH (Group VI) exerted no marked effect on either the thymus weight or adrenal cholesterol and ascorbic acid content, though there was a slight increase in the weight of the adrenals. The changes observed in the corresponding fasted group (VII) closely paralleled those found in the fasted and NaCl-injected rats (Group III). The animals submitted to both cold and fasting (Group VIII) showed the highest degree of thymolysis and adrenal enlargement. These values do not differ significantly from those of the fasted ACTH-treated (15 mg.) group (V). The depletion of adrenal cholesterol and ascorbic acid was maximal in group V. Contrary to the cholesterol, the adrenal ascorbic acid concentration of the fasted ACTH-treated (0.1 mg.) animals (Group VII) was not significantly lower than that of the corresponding controls (Group III).

The histological material (three animals per group) examined in this experiment was somewhat limited, yet, it seemed to indicate that cold (Group VIII) and fasting (Group III) cause an intense discharge of sudanophilic substance from the adrenal cortex, but the administration of ACTH to fasted animals (Groups V and VII) during the same period, does not produce that discharge, even at the high dosage level.

DISCUSSION

The data presented above give at least a partial answer to the points we set out to investigate. In agreement with Sayers *et al.* (1944) we found that a single injection of ACTH produced an initial depletion of sudanophilic material rapidly followed by a return to normal or above normal levels. Repeated injections of ACTH, on the other hand, caused no decrease in the stainable lipid content of the adrenal. This confirms previous observations by Bergner and Deane (1948) and Sayers and Sayers (1948, 1949). Apparently, the depletion of adrenal sudanophilic material is diminished or even reversed after several ACTH injections. In contrast to the picture observed after multiple doses of ACTH, exposure to systemic stress (cold, fasting, spinal cord transection) for the same period, produced an intense discharge of adrenal lipids. Both stressed and ACTH- treated animals showed, however, a comparable discharge of adrenal cholesterol and ascorbic acid, which would indicate a corticotrophic stimulation of the same order. A similar dissociation of the sudanophilia from the cholesterol and ascorbic acid content of the adrenal has been observed in a previously published experiment (Skelton, Fortier, Selye, 1949). This is in agreement with the results of other investigators who have found, using chemical methods of lipid determination, that such lipid constituents as neutral fat (Popjack, 1944)

and phospholipid (Knouff *et al.*, 1941, Ludewig and Chanutin, 1946, Popjack, 1944) may remain unaltered when the cholesterol content is markedly reduced.

It appears from the evidence presented that the action of alarming stimuli on the adrenal cortex cannot be ascribed solely to an increase in ACTH-production. It is suggested that an unknown peripheral factor, present during the alarm reaction, conditions the response of the adrenal cortex to corticotrophic stimulation so that a persistent discharge of stainable lipids ensues.

SUMMARY AND CONCLUSIONS

The effects on the adrenal cortex of single and repeated injections of ACTH were investigated and compared with the changes observed after exposure to acute systemic stress (cold, fasting, spinal-cord-transection). A single injection of ACTH produced a rapid but very transient fall of adrenal stainable lipids. No such decrease was observed after 24 and 48 hours of repeated injections despite a marked depletion of the cholesterol and ascorbic acid content. This is in distinct contrast to the pictures observed after systemic stress of the same duration where all the above adrenal constituents decreased proportionately. It is concluded that ACTH alone can not account for the adrenal response during the alarm reaction.

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SOME BIOLOGICAL PROPERTIES OF DESOXYCORTICOSTERONE GLUCOSIDE[1,2]

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THE GLUCOSIDE of desoxycorticosterone (DCG) has been prepared by Miescher, Fischer and Meystre (1942, 1943) and is of considerable interest and possible usefulness because of its water solubility. Such a substance can be given intravenously or if given by other means a faster absorption should be expected than with the commonly used desoxycorticosterone acetate in oil (DCA). DCG is soluble to the extent of 1 per cent under proper temperature conditions in an aqueous solution of 10 per cent acetamide and 10 per cent glucose. We have made and report here semi-quantitative studies on the activity of DCG in rats under various conditions in which DCA is known to be effective. Other work with the compound has appeared (Meier, Gysel and Mueller, 1944; Laszt, 1946; Greep and Deane, 1947; Gross, 1946, 1948; Herrold, *et al.*, 1948; Cosmos, Duell and Gaunt, 1948; Ravault, Pont and Fraisse, 1949).

SCOPE AND METHOD OF EXPERIMENTS

The activity of DCG and DCA in maintaining life were tested in 50 to 70 gm. adrenalectomized rats fed Purina Laboratory Chow. One lot of this chow was analyzed and found to contain 0.23 per cent sodium and 0.94 per cent potassium. Less DCA was required for the maintenance of adrenalectomized rats in earlier experiments by our group (Eversole, Gaunt and Kendall, 1942; Eversole and Gaunt, 1943) when a different diet containing more sodium and less potassium was used. Controls were observed simultaneously with treated animals and received either no treatment or amounts of an acetamide-glucose solution equal to that given as a stabilizer of the DCG solution. All adrenalectomized animals were discarded which did not succumb when therapy was discontinued. DCG was given at a concentration of 10 mg. per ml. DCA and DCG were given in some cases in equal amounts; in other cases, doses were calculated so as to provide roughly equimolecular quantities of desoxycorticosterone (i.e. 1.3 mg. DCG = 1 mg. DCA).

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TABLE 1. AVERAGE FIGURES ON THE RESPONSE OF ADRENALECTOMIZED RATS TO DESOXYCORTICOSTERONE GLUCOSIDE

Series No	No. rats	Treatment	Wgt. change during treatment, gm./day	Mean survival days	Per cent dying during treatment	Initial wgt., gms.
		DCG given sugcutaneously for 10 days				
1	15	DCG: 1 mg.—one injection/day	+0.9	8.6	73	66
2[1]	7	DCG: 1 mg.—one injection/day	+2.5	14.0	0	64
3	14	DCG: 1 mg.—two injections/day	+1.8	9.9	50	66
4	12	10 mg. Acetamide+10 mg. glucose—one injection/day	−1.0	4.7	100	65
5	4	10 mg. Acetamide—two injections/day	−0.5	6.0	100	66
6	21	Adx. only—controls for Ser. 1–5	−0.0	6.6	—	64
7	10	DCG: 2.5 mg.—one injection/day	+2.7	15.8	0	58
8	11	DCG: 2.5 mg.—two injections/day	+3.0	16.0	0	58
9	8	25 mg. Acetamide+10 mg. Glucose/day	−0.2	5.5	100	57
10	18	Adx. only—controls for Ser. 7–9	+0.2	7.1	—	63
		DCG given by stomach tube for 10 days				
11	6	DCG: 1 mg./day	−0.8	5.0	100	64
12	7	DCG: 2.5 mg./day	+1.0	6.4	100	61
13	9	DCG: 5 mg./day	+2.3	12.2	33	60
14	5	DCG: 10 mg./day	+4.0	14.2	0	67
15	16	50 mg. Acetamide+50 mg. Glucose/day	−1.7	5.0	100	64
16	13	Adx. only—controls for Ser. 11–16	−0.4	5.3	—	60
17	5	DCA: 2.5 mg./day	+2.6	13.2	20	62
18	4	P-nut oil: 0.5 cc.—control for Ser. 17	−2.1	3.8	100	62
		DCG and DCA given subcutaneously for 5 days				
19	3	DCG: 5 mg./day	+4.2	10	0	66
20	3	DCA: 2.5 mg./day	+6.0	14	0	67
		Non-effect of acetamide-glucose solution subcutaneously in normal intact rats				
21	7	50 mg. Acetamide+50 mg. Glucose/day	+3.6	—	—	59
22	7	Untreated controls	+3.8	—	—	60

[1] "Atypical" group—responded to same treatment better than Series 1.

The protective action of DCG was studied in adult adrenalectomized rats against four stresses known to be responsive to DCA: water intoxication, intraperitoneal glucose shock, exposure to cold and potassium intoxication. In these experiments attempts were made to administer DCG in such ways as to reveal whether, because of its water solubility, it would act in a crisis faster than DCA. Appropriate controls were observed simultaneously in all cases.

RESULTS

Solubility of DCG. As others had found, it was necessary to keep the DCG preparation warm in order to prevent precipitation from the acetamide-glucose solution. In cool weather occasional precipitation occurred even in sealed ampules. Once an ampule or vial had been opened or punctured, it was necessary to keep it in an oven at about 40° C. and to handle it in a warm syringe. If precipitation did occur, heating to 70° would bring the substance back into solution.

Maintenance of Adrenalectomized Rats with DCG (Table 1). (a) *Subcutaneous Administration.* Young rats were treated with various daily doses of DCG for 10 days from the time of adrenalectomy. A daily dose of 1 mg. given subcutaneously exerted a barely detectable beneficial effect on growth and survival in most animals (Series 1); in one series for reasons not known this 1 mg. dose was more effective than on any other occasion in which it was observed (Series 2). Two and one-half mg. per day had a distinctly beneficial effect (Series 7). Results with a small series suggested that it takes about 5 mg. per day to maintain a normal growth rate (Series 19).

The effectiveness of 1 mg. doses was slightly enhanced when given in two injections twice daily (Series 3), considering Series 1 as the standard for comparison; the response to 2.5 mg., however, was not clearly improved by dividing the dosage (Series 8). Division of dosage is, therefore, of slight if any benefit.

(b) *Oral Administration.* DCG was effective when given in relatively large amounts once daily by stomach tube. By this route 1 mg. was ineffectual, 2.5 mg. questionably active, and both 5 and 10 mg. highly active (Series 11–14). While precise quantitative statements cannot be made, it would appear that about half of the activity of DCG is lost when it is given by mouth as compared with subcutaneous administration. It is apparent from previous reports (Kuizenga *et al.*, 1940; Spanhoff, 1940; Eversole and Gaunt, 1943) that a much greater percentage of the activity of DCA is lost when it is given orally. In fact, some previous workers (e.g., Eversole and Gaunt, 1943) failed to detect any activity of DCA when given in single daily doses by stomach tube. This finding probably was due to inadequate dosage, because as seen from Series 17, 2.5 mg. of DCA was active when given in single daily doses by stomach tube. Previous workers had used smaller amounts.

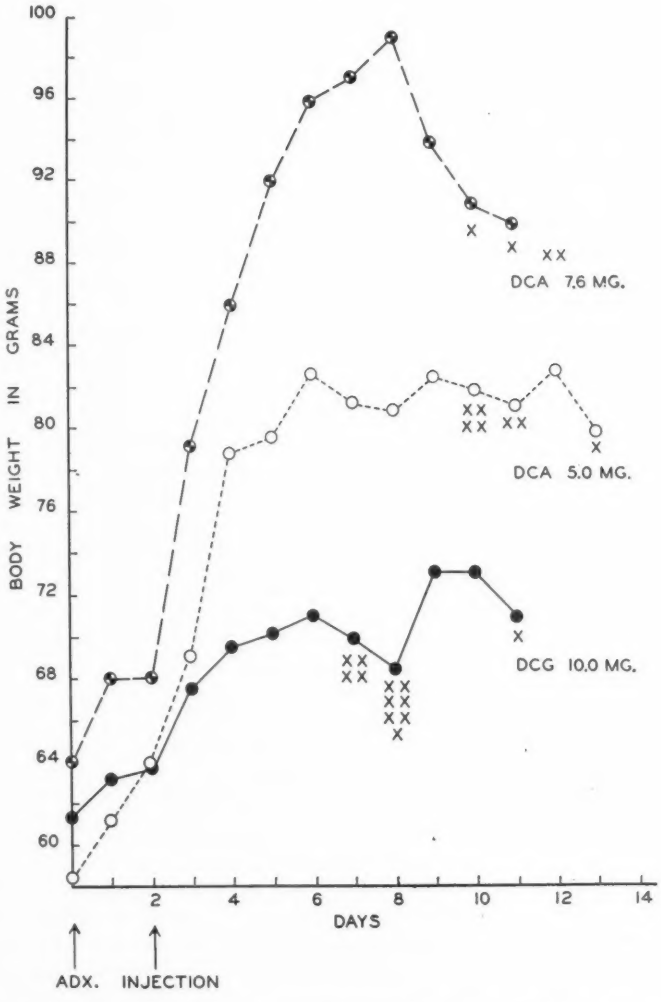

FIG. 1. Survival and growth of adrenalectomized rats given one large dose of either DCA or DCG at 48 hours after adrenalectomy. The effect of DCG was of smaller magnitude and duration than that of DCA. The arrow indicates when the materials were injected. The "X" marks show when deaths occurred and their total indicates the number of animals used.

Duration of Effect of a Single Injection. When a single large dose of DCG (10 mg.) was given on the second day after adrenalectomy, the extent and duration of its effect was much less than that of 5 or more mg. of DCA (Figure 1).

Revival from Crises of Adrenal Insufficiency. Attempts to make quantitative comparisons of the effectiveness of DCG and DCA in the revival of rats from crises of adrenal insufficiency were difficult and largely unsuccessful. No suitable criteria were found by which to identify comparable stages of such crises in different animals, and hence to make injections at comparable times. It was found, however, that if adrenal insufficiency progressed to the point where locomotion was obviously impaired, that 5 mg. of either DCG or DCA would bring about recovery after intraperitoneal injection in some cases. It was not apparent that one form of the hormone was superior to the other.

"Anesthetic" Effect of DCG. The immature rats shown in Figure 1, which received a single subcutaneous dose of 10 mg. of DCG 48 hours after adrenalectomy, passed into a profound coma within 5 to 10 minutes after injection. The initial impression was that the animals were dying, respiration at times being hardly detectable, but within 30 minutes to 2 hours all had regained consciousness and apparent normal activity. A similar result was seen at a later repetition of the experiment in 4 of 7 animals given 10 mg. of DCG subcutaneously and in 5 animals given the same treatment intraperitoneally. One of the latter group failed to recover. Larger adrenalectomized rats (90 grams or more) showed no anesthetic response to 10 mg. of DCG (7 cases). DCA in 5 mg. doses did not produce anesthesia despite its greater potency by other standards than the anesthetic doses of DCG. Ten mg. of DCA, however, given subcutaneously did produce anesthesia in 3 of 11 immature adrenalectomized rats. The coma of animals receiving DCG was not due to an action of acetamide, 100 mg. of which was without apparent effect when administered similarly in 8 adrenalectomized animals. There was no anesthesia when 10 mg. doses of DCG were given by stomach tube in Series 14. Presumably the phenomena observed were manifestations of the anesthetic action of steroids as described by Selye (1941) and the greater effect of DCG than DCA was probably due to a more rapid absorption of the former. Meier, Gysel and Mueller (1944) have seen similar anesthetic effects when DCG in large doses was given intravenously to rabbits.

DCG in Water Intoxication. It is known that DCA is helpful, although less effective than other adrenal steroids, in maintaining water diuresis and protecting against water intoxication in adrenalectomized animals (review, Gaunt 1946). The effects of DCG were compared in this test with those of DCA. Animals received 5 hourly doses of water (3 ml. per 100 sq. cm. of body surface) 18 hours after

adrenalectomy. Intravenous injections of DCG were made under light ether anesthesia in the femoral vein; control animals received the same anesthetic and exposure and manipulation of the femoral vein. Other details were as in previous related experiments (Gaunt, 1944). Injections were made in various times and ways in an attempt to find if DCG, because of its solubility properties, would exert a quicker action by any means of administration than DCA in oil. For instance, it was thought that if the two substances were given

TABLE 2. EXCRETION OF EXCESS WATER BY ADRENALECTOMIZED RATS GIVEN VARIOUS TREATMENTS. (ALL RATS RECEIVED 5 HOURLY DOSES OF WATER BEGINNING AT 0 HOUR

Series	No. of rats	Injection route	Treatment	% water excreted at: 2nd hr.	% water excreted at: 5th hr.	Division of hormone dosage
1	12	Subcu.	5 mg. DCG	21.5	42.8	Half 30 min. before water; half at 2.5 hours.
	12	Subcu.	5 mg. DCA	31.1	51.0	
2	11	Intraven.	5 mg. DCG	17.6	35.9	One dose, 45 min. before water.
	11	Subcu.[1]	5 mg. DCA	23.3	44.0	
3	16	Subcu.	5 mg. DCG	23.0	48.0	One dose, 30 min. before water.
	15	Subcu.	50 mg. A-G[2]	16.2	17.4	
	15	None	None	22.0	28.1	
4	17	Intraper.	6.6 mg. DCG	23.0	41.9	5 doses, given with each dose of water.
	17	Intraper.	5.0 mg. DCA	28.3	50.1	
5	11	Subcu.	6.6 mg. DCG	27.3	48.8	As in Series 4.
	12	Subcu.	5.0 mg. DCA	24.2	45.6	
6	10	Intraper.	65 mg. A-G[2]	14.7	24.6	As in Series 4.
	9	None	None	22.5	29.8	
			Normal, intact animals			
7	12	Subcu.	50 mg. A-G[2]	14.7	60.5	One dose, 30 min. before water.
	12	None	None	22.5	57.9	

[1] Anesthetized and femoral vein manipulated as in group injected intravenously.
[2] A-G = acetamide-glucose, used as stabilizer for DCG. Figures indicate amounts of each substance given.

at the beginning of a water intoxication test that DCG, because of more rapid absorption, might show increased diuresis at earlier stages than DCA.

Results are shown in Table 2. There was no instance in which DCG was clearly more potent than DCA. On the contrary, slight differences were usually in favor of DCA. These findings, together with those described in connection with other stresses below, indicate that the limiting factor in the action of the desoxycorticosterones in such conditions is neither that of solubility nor presumably of absorption, but rather inherent limitations in the potentialities of the compounds themselves.

Other Stresses. When stresses other than water intoxication were studied results of a generally negative type were obtained. Data, therefore, are not given in detail, but the experiments will be noted briefly in case the information might be useful for further work.

(1) *Potassium Intoxication.* Intraperitoneal KCl solution was given to 44 adult rats which had been adrenalectomized for 18 hours. Dosages of KCl were either 0.3, 0.4, or 0.5 ml. of 10 per cent solution per 100 gm. body weight. At the lower dose level survival exceeded 6 hours; the two higher doses killed all animals within 30 to 105 minutes. The administration of 5 mg. of DCG intravenously or intraperitoneally 15 minutes before the KCl injections did not prolong the life span. The same was true in 8 cases which received lethal amounts of KCl by stomach tube. Presumably DCG, like DCA (Lowenstein and Zwemer, 1943), would have been helpful had pre-treatment of some duration been tried. Our purpose here was to see if it would work quickly in a crisis. The results were clearly negative.

(2) *Intraperitoneal Glucose Shock.* When DCA is given prophylactically it is known to afford increased resistance to the shock-inducing effects of intraperitoneal glucose injections in adrenalectomized animals (Remington, 1943). Five mg. of DCG given intravenously three hours after the injection of 10 ml. per 100 gm. body weight of 5.5 per cent glucose intraperitoneally did not hasten recovery (5 cases) more than 5 mg. of DCA given subcutaneously (5 cases).

(3) *Exposure to Cold.* Forty-eight rats, adrenalectomized for 18 hours, and averaging 94 gm. body weight, were placed in a refrigerator at 5° C. and left until death. Cases receiving 5 mg of DCA survived slightly longer than those given 5 mg. of DCG and the latter in turn slightly longer than untreated controls. Differences, however, were not clearly significant.

Possible Toxicity of Acetamide. Acetamide, used as a stabilizer in the DCG solution, is known as a non-toxic substance in normal individuals—a conclusion consistent with all of our data (e.g., Table 1, Series 21 and Table 2, Series 7). Adrenalectomized animals, however, show sensitivity to many agents not usually considered toxic. In most of the above experiments, therefore, controls receiving acetamide-glucose solution as well as untreated controls were run. There were instances in which acetamide seemed to be a slightly deleterious agent but such effects were neither marked nor consistent. As mentioned in an earlier section, injections of single doses of 100 mg. of acetamide into immature adrenalectomized animals were without obvious effect. It is concluded therefore, that the acetamide stabilizer was not responsible for the relative weakness of DCG under the conditions of our experiments.

Fate of DCG at Injection Sites. One possible reason for the lack of rapid action of DCG as seen in some of these experiments was that it

might precipitate at an injection site or in the blood stream. Such an explanation is improbable as indicated by two observations. First, when 10 mg of DCG was injected subcutaneously, dissection 10 to 30 minutes later revealed no visible precipitate at the injection site. This was obviously an inconclusive observation but more definite was the fact that when DCG was added to rat blood plasma *in vitro* (10 mg. in 1 ml. solvent per 2 ml. of plama) there was no microscopically detectable precipitation when the plasma was kept at body temperature. When temperature was reduced an obvious precipitation occurred.

DISCUSSION

It is readily apparent that DCG does not approach the effectiveness of DCA in the maintenance of life and growth in rats. Data presented here plus that already in the literature would suggest that when given in single doses DCA in oil is somewhere in the order of 5 times as active as DCG. Such findings are roughly comparable to those of Meier, Gysel and Mueller (1944) who found the dog to require 2 to 4 times as much DCG (in divided doses) as of DCA. This might be expected of a water soluble substance quickly absorbed and utilized. The relatively evanescent effects of a single large dose of DCG (Fig. 1) as compared with DCA, and the fact that DCG exerts anesthetic effects more readily than DCA, suggests a rapid absorption.

In view of these facts it is surprising that no situation was found in our cases of stress-induced crises in which DCG would act more quickly and effectively than DCA in oil. This was presumably not due to any more rapid inactivation of DCG than DCA by the liver. The fact that the ratio of the effective oral:parenteral dose of DCG is lower than for DCA would imply that if there is any difference in the rate of hepatic inactivation of the two compounds it would be in the opposite direction.

It is probable, therefore, that the limiting factor in the action of the desoxycorticosterones in such experiments is not the rate of absorption but inherent limitations in the properties of the compounds themselves.

From these experiments it would seem that little can be done in the rat with DCG that cannot be done with DCA in oil. The fact that DCG can be given intravenously, however, and without the complications of an oil vehicle may in itself be highly advantageous in various situations. In general, higher doses of DCG than DCA would probably be needed for any equivalent action. There are, however, limitations to the amounts of DCG that can be given at one time in rats because of the possibility of anesthetic actions.

Generalizations of this type must be limited at the present time to the rat. Meier *et al.* (1944), Herrold, *et al.* (1948) and Swingle

Perlmut and Seay (personal communication) have made extensive studies on the effects of DCG in the maintenance of adrenalectomized dogs, and in revival from crises of adrenal insufficiency. They have found it to be a highly effective substance in revival and while exact comparison is not possible, DCG appears in general to work better in the dog than in the rat.

SUMMARY

Desoxycorticosterone glucoside (DCG), a water soluble compound, maintained life and growth in young adrenalectomized rats when given by daily subcutaneous injections but dosages needed were much higher than for desoxycorticosterone acetate in oil (DCA).

DCG was about one-half as active when given in single daily doses by stomach tube as by subcutaneous injection.

Single large doses of DCG exerted effects of less magnitude and duration than did DCA.

Large doses of DCG exert a profound "anesthetic" effect in adrenalectomized rats.

DCG helped protect adrenalectomized rats from water intoxication but did not exert more rapid actions than DCA in oil.

In other stresses—exposure to cold, intraperitoneal glucose shock and potassium intoxication—DCG showed little if any protective action when given by various routes during or just before application of the stress. This indicates that the limitations of desoxycorticosterone in such conditions is not due to slow absorption.

Evidence from other laboratories indicates that DCG is a generally more effective substance in dogs than in rats.

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THE SIMULTANEOUS DETECTION OF THYROID AND THYROTROPHIC HORMONES IN VERTEBRATE SERA

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ALTHOUGH it is generally accepted that reciprocal regulatory control exists between the thyroid and pituitary glands (Aron, 1930; Loeb, Bassett, and Friedman, 1930; Houssay, Biasotti, and Magdalena, 1932; Kuschinsky, 1933; VanEck, 1939; Uotila, 1940; Galli-Mainini, 1941; Rawson and colleagues, 1942, 1943) the inadequacy of present methods for the detection of thyroid stimulating hormone (TSH) in the circulating fluids continues to thwart complete understanding of anterior-pituitary-thyroid gland interaction in the vertebrate organism. Assays of pituitary thyrotrophin have been done extensively and with reasonably consistent results (Adams, 1946), nevertheless, these give no measure of the amounts of TSH released into the circulation. Aside from the recent reports of DeRobertis (1948) and Purves and Griesbach (1949), there is little information available which accurately defines the levels of thyrotrophin in blood in either normal, experimental, or clinical conditions. Bioassays utilizing treated as well as untreated sera have yielded discrepant results (Aron, VanCaulert and Stahl, 1931; DelCastillo and Magdalena, 1931; Hertz and Oastler, 1936; Cope, 1937, 1938; Collard *et al.*, 1940; Dvoskin, 1948), and doubt exists as to whether or not the presence of TSH in blood has been indisputably shown (Albert, 1949). The occasional successes which have been reported for the detection of thyrotrophic hormone in the circulating fluids, moreover, generally have required preliminary extraction of the thyroid hormone fraction and concentration of the thyrotrophin fraction of blood before any appreciable activity of the latter becomes manifest (Bodart and Fellinger, 1936; Fellinger, 1936; DeRobertis, 1948). Since the activity of thyrotrophin in blood is believed to be conditioned by a complexity of factors, among which, thyroid hormone may be especially important, it is questionable whether or not procedures which alter the hormonal balance prior to bioassay actually lead to a true

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measure of the *effective* TSH in circulating fluids. There is urgent need, consequently, for a method of assay which is sufficiently sensitive to detect simultaneously both components of the thyroid-TSH balance in untreated blood.

The suitability of the stasis (starved, non-metamorphosing) tadpole as a test object for the bioassay of pituitary thyrotrophin has already been demonstrated (D'Angelo, Gordon, and Charipper, 1942). Experiments have also indicated the applicability of the stasis tadpole method for the detection of TSH in blood (Gordon, Goldsmith and Charipper, 1945; D'Angelo and Gordon, 1949; D'Angelo, Gordon, Paschkis, and Cantarow, 1949). The present work will describe the results of experiments establishing the validity of the stasis tadpole as a test object for the simultaneous detection of thyroid and thyrotrophic hormones, and, in addition, will consider the results and implications of a study of the thyroid-TSH balance in vertebrate sera as determined with the method.

MATERIALS AND METHODS

The basic procedures and rationale underlying the use of the frog tadpole (Rana pipiens) for TSH assay have been given in detail (D'Angelo, Gordon and Charipper, 1941, 1942). The method takes advantage of the fact that total starvation of the tadpole at early hindlimb length stages (3–5 mm.) induces thyroid gland atrophy and metamorphic stasis. The metamorphic arrest can be overcome in such "stasis" animals by the administration of either TSH or thyroid hormone. The specificity of the effect is easily differentiated by direct examination of the thyroid gland.

The frog larvae are obtained initially, either in the field, or in the laboratory (at all times save the late summer months) by experimentally induced ovulation and fertilization (Rugh, 1934, 1948). The tadpoles are reared in tap water[2] or spring water, are maintained at mildly fluctuating room temperatures (22° ± 3°c.),[3] and fed ample quantities of boild spinach until the 3–5 mm. hindlimb stage is attained (4–6 weeks after fertilization). At this time all food is withdrawn, and animals are kept on this starvation régime throughout the ensuing experimental period.[4]

In the present study, the detection of pituitary TSH was re-quantitated utilizing the International Gland Preparation of desiccated ox anterior pituitary.[5] A stock standard was prepared by introducing 107 mgm. of the powder into 50 cc. of saline (to pH 8–8.5 with 0.1 N NaOH). Serial dilutions

[2] Tap water drawn through newly-installed plumbing is usually toxic.

[3] Sustained temperatures higher than these should be avoided since mortality in injected animals may be appreciable, and developmental rates higher than those at the lower laboratory temperature.

[4] It is important that the starvation be initiated at the 3–5 mm. hindlimb stage since animals adequately fed to more advanced stages may continue their metamorphosis and show histologic evidences of thyroid gland activity despite the inanition. This complication may be circumvented by deferring injection of the test animal for several days so that an occasional tadpole which advances in metamorphosis may be discarded.

[5] Obtained through the courtesy of Dr. A. B. Nichols from the committee of Revision, U. S. Pharmacopoeia. Five daily doses of 10 mgm. each will cause approximately 50% increase in the thyroid weight of immature female guinea pigs (225 grams).

of the resulting fine suspension were then made so that test animals in experimental groups received a total of 1.5, 7.5, 75.0, 150.0 or 750.0 micrograms of the pituitary standard administered over 7 intra-peritoneal injections. The injections (0.05 cc.) were made through a 26 or 27 gauge needle, and were given on alternate days. Approximately 24 hours after the last injection, hindlimb extension was measured to the nearest millimeter, other developmental criteria noted, and the animals were then preserved in the fixative (Bouin's picro-formol).

Since the thyroid gland of the stasis tadpole is too small (200–300 micra) for convenient removal, a decapitation method was employed. The fixed tadpole head was trimmed by transverse cuts to a region just anterior and posterior to the eyes and this portion of the head was then carried through the dioxan-paraffin method and serially sectioned in the transverse plane at thicknesses of 5 micra. Sections containing the thyroid lobes were stained with hematoxylin and eosin, or with the Masson technique. Measurement of the thyroid cell height was done by slight modification of the basic microhistometric techniques employed for the guinea pig and chick thyroid (Rawson and Starr, 1938; Rawson and Salter, 1940). The acinar cell height was measured under oil immersion (2 mm. objective) with an ocular micrometer. Two thyroid cells whose borders could be clearly delineated were measured in each definitive follicle of every 8th section throughout one thyroid lobe. The two thyroid cells were chosen at opposite poles of the follicle along any one diameter. In this manner, representative cells of all follicles in the gland were considered. The number of cells measured in any one thyroid gland ranged from 50–150 depending on the degree of hyperplasia with TSH.[6]

In all experiments which were designed to detect thyroxine or TSH in the circulating fluids, the serum used was obtained without chemical or fractionation procedures from the pooled bloods of at least several animals in any donor group. In laboratory animals (rat and guinea pig) blood was drawn under light anesthesia by direct cardiac puncture. Beef and lamb blood were procured from freshly slaughtered animals at the abbatoir, and that from the horse and human being by venipuncture. The bloods of lower vertebrates were best obtained from the neck vessels after decapitation. Sterile precautions were taken at all times. Sera were always refrigerated except when allowed to thaw just prior to the injection period.[7]

The effects of d,l-thyroxine (Hoffman-LaRoche) on the stasis tadpole were quantitatively studied by determining the activity of small amounts of this hormone when added to normal guinea pig serum (females, 300–350 grams body weight). The standard stock solution (1–100,000) was added to aliquot parts of the serum in amounts to produce thyroxine concentrations in sera equivalent to 1, 10, 100, and 500 gamma per cent. Test animals received 3–7 injections of 0.05 cc. of the "thyroxinized" sera on alternate days. The effects obtained with the thyroxinized sera were compared with those resulting from normal serum and from serum of guinea pigs rendered goitrous

[6] The stasis thyroid is invariably "resting," and the acinar cell height, represented almost entirely by the condensed nucleus, shows little variation. Bouin's fixative is excellent for proper histologic preservation of the amphibian thyroid cell. The importance of using the same fixative in studies involving measurement of the thyroid epithelium has been pointed out (Wilcke, 1935).

[7] Under these conditions the sera were well maintained. Contamination occasionally occurred, however, and such sera proved to be toxic in the test animal.

by long term administration of propylthiouracil in the ration (D'Angelo, unpublished). Control injections of the goiterogen alone (400 mgm. per cent) were also made. The possible participation of inorganic iodine "per se" in the metamorphic advance of test animals with sera was investigated by the administration of Lugol's solution (5% elemental iodine, 10% KI), and elemental iodine alone 5%). Injection of these solutions in full strength proved to be toxic to the stasis tadpole but dilutions of 1–200 were tolerated. As a preliminary experiment to the simultaneous detection of thyroxine and TSH in blood, the effects of these principles were first determined separately and then together as a mixture in the stasis animal. Relatively low concentrations were chosen (thyroxine, 10 gamma per cent; TSH, 0.01 Junkmann-Schoeller unit[6]). Equivalent hormone concentrations were then made up in normal human male serum (pooled, ages 21–35) and tested in the same manner by intrapleuroperitoneal injection.

RESULTS

The high order of sensitivity of the stasis tadpole to injected TSH is evident from the data in Table 1. Administration of the International Gland Preparation of desiccated ox anterior pituitary, in

TABLE 1. BIOASSAY OF TSH (INTERNATIONAL GLAND) IN THE STASIS TADPOLE

Dosage	No. animal	Metamorphic changes		Thyroid response
		Hindlimb increase	Body	
750 μg.[1] (0.05JS)	10	6.8±0.4[2]	Imminent Forelimbe	9.5±0.3[3]
150 μg. (0.01JS)	10	4.9±0.3	Constrict. at Forelimb region	8.0±0.2
75 μg. (0.005JS)	10	4.5±0.3		7.6±0.2
7.5 μg. (0.0005JS)	10	2.1±0.1		6.5±0.3
1.5 μg. (0.0001JS)	10	0.3±0.1		4.9±0.1
Control (water)	10	0.5±0.2		4.8±0.2

[1] Total amount of standard powder over 7 injections, and in parentheses the equivalent Junkmann-Schoeller unitage.
[2] Mean hindlimb length increase (mm.) ±standard error of mean. Mean initial hindlimb lengths in all groups were the same (3.5–3.8)
[3] Average mean thyroid cell height in micra ±standard error.

amounts totalling 7.5 to 750 micrograms over the entire injection period, induced morphogenetic changes in the thyroid gland of test animals and re-initiated their metamorphosis. The histologic evidences of thyroid activation, characterized by enlargement of the nuclei and an increase in the amount of cytoplasm of the follicular epithelium followed by liquefaction of the colloid and follicular collapse, were progressively intensified with the higher dosages of pituitary powder used (figures 2, 3, 4, 5). These changes were plainly

[6] Antuitrin T (Parke Davis)—courtesy of Dr. Oliver Kamm.

evident when compared with the inactive thyroid picture of the stasis control. Microhistometric measurements of the thyroid epithelium indicated that the mean cell height was significantly increased with as little as 7.5 micrograms of pituitary material and was progressively elevated with the higher doses. The increases in thyroid cell height with the 7.5, 75, 150, and 750 microgram dosages were respectively 35%, 58%, 66%, and 98% of the control stasis value, and reference to the nomogram (Fig. 1) demonstrates the cell height in-

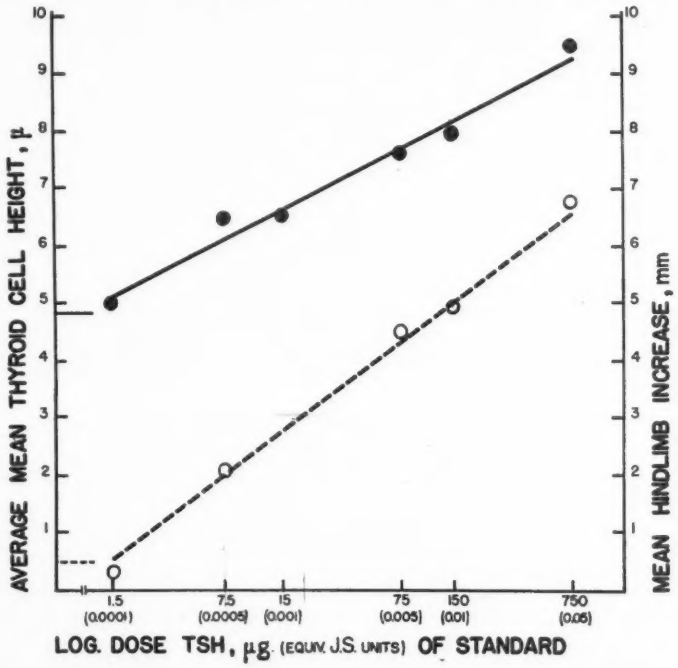

FIG. 1. Log dose-response curves indicating the increase in thyroid cell height (solid line—$Y=1.54x+4.88$), and extension of hindlimb (broken line—$Y=2.26+0.12$) in the statis tadpole with increasing dose of TSH (standard preparation of desiccated ox pituitary). Cell height at each point based on counts in 6–9 animals.

Horizontal bars on ordinate represent values in control animals. Equations for the lines were calculated by the method of least squares.

crease, over the range of TSH tested, to be a linear function of the logarithm of the dose. No significant increase in the mean acinar cell height was obtained with the smallest dose of pituitary powder used (1.5 μg.), and the entire histologic picture of the thyroid in animals receiving this dose was indistinguishable from that in stasis controls. It appears, likely, therefore, that the minimal effective dose of TSH detectable by microhistometric measurement of the follicular epithelium in the tadpole thyroid corresponds to approximately 7.5 μg.

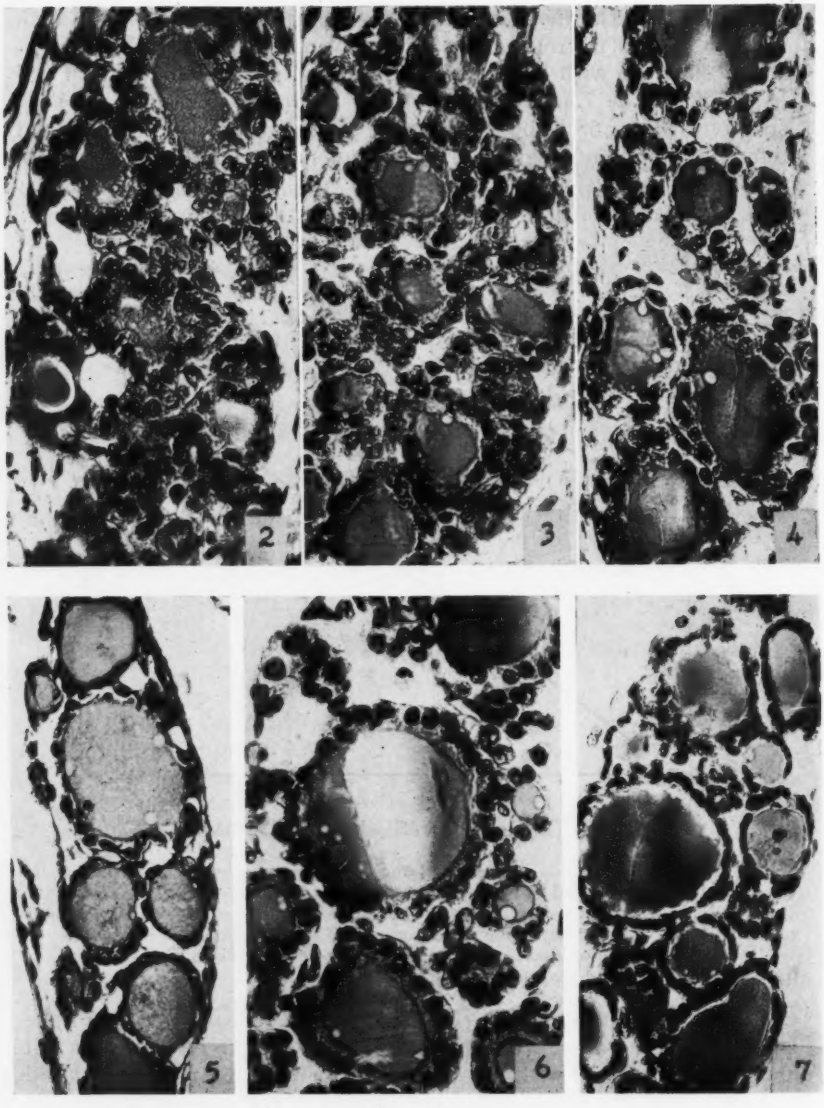

PLATE 1

of the standard powder. Translation of this amount of the standard pituitary preparation into Junkmann-Schoeller units[9] indicates that the stasis thyroid gland responded to 0.0005JS units with a statistically significant increase in mean acinar cell height. Since this unitage was administered to the test animal in 0.35 cc. of fluid, it may be calculated that the stasis tadpole can detect thyrotrophic hormone in concentrations as low as 0.001JS units per milliliter of injected fluid.

It is seen further (Table 1, fig. 1) that the extension of the hindlimb (a sensitive criterion of metamorphic advance) closely parallels the histologic evidences of thyroid activation in animals receiving effective doses of TSH. It is noteworthy that the lowest dose (7.5 μg. which elevates thyroid cell height also effects a significant increase in hindlimb length, and can, thus, cause resumption of metamorphosis in the stasis animal. With higher doses of pituitary substance (150 μg. and 750 μg.) the metamorphosis of the test animal is so accelerated that superimposed on hindlimb extension are other developmental events, such as characteristic changes in shape of the head, constriction of the body at the opercular region presaging emergence of the forelimbs, and emaciation.[10] In contrast, test animals receiving either water injections or amounts of pituitary (1.5 μg.) which fail to stimulate the thyroid gland show no appreciable increase in hindlimb length and remain in a state of arrested development.

The results summarized in Table 2 demonstrate the marked sensitivity of the tadpole to thyroid principle. Three or four injections of 0.05 cc. of normal guinea pig serum to which d,l-thyroxine had been added in dilutions of 1–200,000–1,000,000 (500–100 gamma

[9] 15 mgm. of this preparation is equivalent to 1JS unit (Albert *et al.*, 1946).

[10] These developmental events, together with forelimb emergence and tail resorption, occur in the climax portion of normal metamorphosis at a time when thyroid activity is maximal (D'Angelo, 1941). Such criteria supersede hindlimb extension in prescribing the developmental age of the animal.

All figures are photomicrographs of transverse sections (×380; Bouin, hematoxylin and eosin) through the thyroid gland of stasis tadpoles following the experimental treatment indicated below.

FIGS. 2 and 3. The marked histologic response (cell hypertrophy and hyperplasia, follicular collapse) of the thyroid with 750 μg. (0.05JS units) and 75 μg. (0.005JS units respectively of the standard pituitary preparation.

FIG. 4. The activation changes in the thyroid (nuclear enlargement and cytoplasmic increase in the follicular epithelium) with 7.5 μg. (0.0005JS units) of the standard pituitary preparation.

FIG. 5. The typical morphologically inactive picture (condensed nuclei, flattened epithelium, distended follicles) of the control stasis thyroid gland.

FIG. 6. The pronounced morphogenetic changes in the thyroid gland following the administration of normal rat serum (0.35 cc.).

FIG. 7. The non-effect of hypophysectomized rat serum (0.35 cc.) on the thyroid histology of the stasis animal.

TABLE 2. BIOASSAY OF D,L-THYROXINE IN GUINEA PIG SERUM

Treatment	No. test animals	No. inject.	Metamorphic changes		Mean thyroid cell height
			Hindlimb increase	Body	
Normal Serum	15	7	3.0±0.3[1]		5.1±0.1[2]
Serum and Thyroxine					
500 gamma%	11	3	6.2±0.7	Tail Resorp.	
100 gamma%	11	4	7.2±0.7	Tail Resorp.	4.9±0.1
10 gamma%	11	5	5.0±0.4		5.3±0.1
1 gamma%	10	7	2.8±0.4		
Goitrous Serum	10	7	2.9±0.3		6.8±0.2
Prop.-Thiouracil 400 mgm.%	8	7	0.6±0.1		5.0±0.1
Elem. Iodine 25 mgm.%	10	7	2.0±0.2		4.9±0.1

[1] Mean hindlimb increase in mm. ±standard error of mean. All groups had similar initial hindlimb lengths (3.3–3.5).
[2] Micra ±standard error.

per cent) were sufficient to precipitate metamorphosis to final stages of the transformation process (forelimbs and tail resorption). Equivalent treatment with normal serum alone, however, produced relatively slight, although significant, effects on development. Appreciable effects on hindlimb extension were still obtainable with thyroxnized serum in concentrations as low as 10 gamma per cent, and calculation of the minimal amount of effective thyroxine in test animals indicates that as little as 0.025 microgram of the hormone could be detected.

The developmental advance resulting from the administration of thyroxinized sera did not involve any appreciable change in the resting status of the thyroid in the test animal. Mean thyroid cell heights were not significantly higher than with serum alone, and in either instance approximated those in control animals. It is obvious, thus, that the metamorphic effects of these sera were peripheral, and were not mediated through any major change in the pituitary-thyroid axis of the starved tadpole. The advancement of metamorphosis with either normal or thyroxinized guinea pig sera could not be attributed, moreover, to the total iodine content of the serum *per se*. The injection of iodide or elemental iodine, in amounts exceeding several thousand times the total iodine present in the least effective dose of thyroxine used gave only slight or questionable effects on metamorphosis. It appears, therefore, that the metamorphosing stimulating capacity of guinea pig serum is these instances was attributable to organic iodine, presumably in the form of thyroid hormone. Although no TSH was demonstrable in normal guinea pig serum, it may be seen (Table 2), that thyrotrophic activity is present in the circulating blood of guinea

pigs made goitrous by prolonged treatment with the antithyroid agent, propylthiouracil. The activation changes in the thyroid were appreciable with this experimental serum, and mean cell height (6.8 μ) was significantly elevated above the normal (5.1 μ). In each instance, however, hindlimb extension was essentially the same (to be discussed later). Control injections of the drug alone (in sufficiently high concentration to approximate that which might be present in the fluids of the propylthiouracilized animals) were without effect on either the thyroid histology or the arrested development of the stasis animal.

The results of those experiments which were designed to test simultaneously for thyroxine and TSH in serum are indicated in Table 3. The data reveal that the degree of thyroid stimulation following a standard dose of TSH to test animals was approximately the

TABLE 3. SIMULTANEOUS DETECTION OF TSH AND THYROXINE

Treatment[1]	No. test[2] animals	Metamorphic changes		Mean thyroid cell height
		Hindlimb increase	Body	
Thyroxine	10	3.5 ± 0.1[3]		5.4 ± 0.2[4]
TSH	10	5.7 ± 0.4	Constrict.[5] at fore limb (20%)	7.8 ± 0.2
Thyroxine and TSH	10	7.6 ± 0.3	Fore limb Extrusion (50%)	8.1 ± 0.4
Human Serum (male)	20	5.2 ± 0.3		5.8 ± 0.1
Serum and Thyroxine	20	7.4 ± 0.4	Head Changes (25%)	6.2 ± 0.4
Serum and TSH	20	8.4 ± 0.5	Head Changes (50%)	8.1 ± 0.3
Serum, TSH and Thyroxine	20	7.9 ± 0.4	Fore limb Extrusion (50%)	8.1 ± 0.1

[1] Standard hormone doses (thyroxine, 10 gamma %; TSH, 0.01JS unit).
[2] Each animal received 0.05 cc. at each of 7 injections, on alternate days.
[3] Mean hindlimb increase (mm.) ± standard error, from mean initial length (3.3–3.5 mm. in all test groups).
[4] Average mean thyroid cell height in micra ± standard error.
[5] In parentheses, the percentage of animals attaining the metamorphic events indicated.

same whether the hormone was given as such or whether administered in normal human serum. The thyroid response occurring with simultaneously administered thyroxine and TSH was not significantly different, moreover, from that with TSH alone. In general, the acceleratory effects on the developmental advance of test animals were additive when both hormones were present, so that climactic metamorphic events appeared sooner and more frequently than with either hormone alone. It was evident, therefore, that human serum did not potentiate nor diminish the effects of thyroxine or TSH in the concentrations employed, and that these principles could be detected simultaneously in the same sample of serum with no apparent antagonism or neutralization between them.

The results of the investigation on vertebrate sera (Table 4), are best interpreted by reference to the standard nomogram (Fig. 1). Both beef and rat sera advanced the metamorphosis of test animals appreciably and to almost the same degree as indicated by approximately equal hindlimb increases. The thyroid response, however, differed markedly with each serum. Thyroid cell heights with beef and rat serum were 5.5 micra and 8.8 micra respectively. Extrapolation of these values on the standard curves for acinar cell height and hindlimb extension indicates that the degree of metamorphosis

TABLE 4. THYROID-TSH ACTIVITY IN VERTEBRATE SERA

Serum[1] donor	No. test[2] tadpoles	Mean hindlimb increase (mm.)	Detectable TSH (JS units/millil.)
Beef (4)	20	6.4±0.2	0
Rat (3)[3]	16	5.7±0.3	0.06
Hypx. Rat (3)	20	1.3±0.1	0
Human (3)	20	5.2±0.3	0.0009
Lamb (6)	20	4.2±0.1	0.0009
Horse (2)	20	3.8±0.1	0
Guinea pig (3)	16	3.2±0.3	0
Hypx. G.P. (2)	17	0.6±0.1	0
Pigeon (4)	20	2.0±0.1	0.0009
Turtle (2)	20	1.3±0.1	0
Frog (24)	20	0.9±0.1	0

[1] Serum from pooled bloods; number of donors in parentheses.
[2] Each animal received 0.05 cc. at each of 7 injections.
[3] Male rats at 140–150 grams were used. Hypx. rats were used 6 weeks after operation, with all target organs atrophic.

achieved with beef serum cannot be accounted for by TSH present but must be due largely to circulating thyroid principle (hindlimb extension corresponding to a thyroid cell height of 5.5 micra would not exceed 1 mm.). In contrast, the titer of TSH in rat serum must be high, as indicated by the marked thyroid response (Fig. 6), and the hormone is present in sufficiently high concentration to account for most of the hindlimb extension occurring in test animals. Accordingly, the thyroid-TSH ratio in beef and rat blood must be such that in the former thyroid hormone concentration is relatively high with little TSH present, whereas, in the latter, TSH content is high with thyroid hormone in low concentration. Since in each instance the volume of injected serum was the same (0.35 cc.), it may be calculated that the concentration of TSH in beef serum was less than 0.001JS units per ml., but was considerably higher in the rat (0.06JS units). The results obtained with serum from hypophysectomized rats are consistent with this interpretation since the mean thyroid cell height (4.7 micra) was much below that with normal rat serum (compare Figs. 5, 6, 7), indicating, thus, practically complete absence of TSH in the circulating fluids of the rat following hypophysectomy.

The results obtained with normal guinea pig serum and from that of animals receiving propylthiouracil can be similarly interpreted

from the nomogram. Despite the fact that hindlimb extension with both sera was almost identical (Table 2), the thyroid-TSH balance in each must have been quite different. The hindlimb increase with the experimental serum corresponded to that expected from the degree of thyroid activation found (i.e., from TSH activity alone); with normal serum the metamorphic advance reflected thyroid hormone activity alone (since no TSH was detectable). In effect, the long term administration of propylthiouracil to the guinea pig increases TSH in the circulating blood but concomitantly reduces thyroid hormone so that the net metamorphic advance is no different from that with normal serum.

Human, lamb and horse sera were all effective in initiating metamorphosis in the stasis tadpole but at a lesser rate than beef or rat serum. The metamorphosing effect of these sera, like that in the ox and in the guinea pig, was best accounted for by thyroid rather than thyrotrophic principle. Some TSH was detectable, however, in human and lamb serum since mean thyroid cell height with these were significantly elevated (5.8, 5.7 micra) over the control value (4.8 micra). TSH could not be detected in horse serum. The thyroid cell height was not significantly changed from the stasis control condition. Of the non-mammalian sera tested only that of the pigeon had thyrotrophic activity. The relatively slight hindlimb extension which occurred with this serum corresponded to that expected from its TSH content, as computed from the elevated mean thyroid cell height (5.8 micra) in the test animal. Although the amount of TSH in pigeon serum was relatively low, the status of the thyroid-TSH balance more closely approximated that of the rat than for other mammalian sera (in which thyroid hormone activity is characteristically high). In general, however, the blood of lower vertebrates was least effective in causing metamorphosis, and neither thyroid nor TSH activity could be detected in frog or turtle serum.

DISCUSSION

For no other trophic hormone of the anterior pituitary have investigators found it necessary to employ a greater variety of criteria and test forms than for the bioassay of TSH. Albert (1949) has critically discussed the present status of the problem, and has reiterated the urgent need for a method sufficiently sensitive to detect the small amounts of TSH which may exist in the circulating fluids. Direct comparison of the results of TSH assay in different test forms has generally been invalid since dissimilar preparations were used without reference to some common standard. Utilization of the International Standard Gland Preparation in the present study permits valid comparison between the stasis tadpole method and those on the chick and the hypophysectomized rat. Albert *et al.* (1946), employing a composite method of thyroid weight and cell height increase with de-

creased iodine, have indicated that 15 mgm. of this standard preparation is equivalent to 1JS unit when tested in the day old chick, and at least 0.125JS unit are required to elevate significantly the mean thyroid cell height in this test form (Rawson and Salter, 1940). In contrast, the present study reveals that only 0.0005JS unit are necessary to produce an equivalent degree of thyroid activation measured with similar microhistometric techniques. In the hypophysectomized rat, 20 times as much of the standard preparation is needed to increase significantly the numbers of intracellular colloid droplets in the thyroid cell, and considerably more to elevate cell height (Dvoskin, 1947a, 1948).

Although the standard pituitary powder has not been used in the cytologic test of DeRobertis and DelConte (1944) on the guinea pig, its sensitivity to TSH (0.0002–0.001 arbitrary unit quadruplicates the number of intracellular colloid droplets) appears to be no greater than that demonstrated microhistometrically in the starved tadpole. That cytologic events in activated thyroids precede histologic ones can hardly be denied; nevertheless, caution must be exercised as to the specificity of such criteria for TSH assay. Droplet formation can be induced in both chick and hypophysectomized rat with various toxic agents and in the absence of TSH (Dvoskin, 1947B, 1948), moreover, colloid continues to form in the thyroids of hypophysectomized animals (B. M. Allen, 1929; Astwood *et al.*, 1943).

Use of the stasis tadpole method for TSH detection obviates several important objectionable features generally ascribed to other methods. The activation changes in the thyroid have so far proved to be specific for material of pituitary origin. The morphologic picture of the thyroid in the test animal is essentially the same at any period of the year, unlike that in the chick in which aperiodic variations, temperature effects, and the seasons render the chick unit a "freely floating variable" (Albert, 1949). The histologic response of the tadpole thyroid is easily interpreted and measured because of its relatively small size. The precision of the method is attested to by the fact that the thyroid response is a linear function of the logarithm of the TSH dosage, over the wide range tested. The question may be justifiably posed, however, as to its convenience. The administration of test material over a period of days postpones direct examination of the thyroid gland even though metamorphic changes become evident. Although the detection of TSH can be accomplished by a single injection without considering the secondary effects on metamorphosis, it is precisely the metamorphic phenomenon which, when correlated with the presence or absence of thyroid stimulation, renders the tadpole a unique test object in that both thyroid and thyrotrophic principles can be bioassayed simultaneously.

The validity of an assay method which purports to determine simultaneously both thyroid and thyrotrophic hormones in blood

must bear close scrutiny. Many experimenters, utilizing either thyroid feeding or immersion in thyroxine solutions, have confirmed the original observation of Gudernatsch (1912) that thyroid substance induces the precocious metamorphosis of tadpoles (see B. M. Allen, 1929, 1938, for detailed reviews). Although it is possible to cause the metamorphosis of tadpoles with di-iodotyrosine or even inorganic iodine, the amounts required are considerably greater than necessary if the element is incorporated into the thyroxine molecule. Under the quantitative circumstances of the stasis test as described, it is quite likely that the metamorphosing capacity of serum is a measure of its thyroid principle provided TSH activity has been ruled out. The failure of either Lugol's or elemental iodine solution to advance appreciably the development of the stasis animal, despite high concentrations, supports this inference. That di-iodotyrosine may contribute to the "thyroid" activity of an unknown sample appears equally remote since the effective concentration required to induce metamorphosis in the stasis tadpole is far greater than that in body fluids (Money, personal communication).

It is becoming clear that the concept of reciprocal regulatory control does not explain all observable phenomena in thyroid-pituitary interaction. Although exogenous thyroid substance in the intact animal induces a resting thyroid gland which can be correlated with reduced pituitary TSH (Cameron and Carmichael, 1921; Kuschinsky, 1933; Hohlweg and Junkmann, 1933; Reforzo-Membrives, 1943; Adams and Jensen, 1944), thyroid hormone inhibits the histologic response to simultaneously administered TSH in the hypophysectomized (VanEck, 1939; Cortell and Rawson, 1944) as well as the intact animal (Loeb, Bassett, and Friedman, 1930; Loeb, 1932; Aron *et al.*, 1931; Loeser and Thompson, 1934). Hyperplastic thyroids can occur in hypophysectomized rats with lowered iodine intake (Chapman, 1941), and iodide excess inhibits the morphogenetic changes in the thyroid of the hypophysectomized animal with TSH (Rawson, 1949). TSH, moreover, can be inactivated by elemental iodine in an oxidative reaction (Albert *et al.*, 1946) which can conceivably occur in the body (Wright and Trikojus, 1946). The evidence, thus, is highly suggestive that the TSH activity in blood may be profoundly influenced by circulating thyroid hormone and iodine levels. The suitability of the stasis tadpole method for the bioassay of both thyrotrophic and thyroid principles in blood, where the existing hormonal balance has not been altered by chemical manipulation, should provide a more advantageous approach to the general problem of thyroid-pituitary interaction.

It is evident from this study that appreciable differences in the thyroid-TSH balance may be found in the body fluids of various vertebrates. If it is tentatively assumed that blood TSH is the same substance as pituitary thyrotrophin, it would appear that in all species

tested, except the rat, the TSH concentration does not exceed 0.001 JS units per milliliter of serum, and may be much less. Just how the total concentration of TSH is partitioned among the various blood components is not yet known although serum apparently plays a dominant and exclusive role in the transport of thyroxine by blood (Zavadovsky and Novikov, 1926). The results of TSH assay of blood are consistent, in the main, with those showing the order of pituitary TSH content in various species (Adams, 1946). In both the pituitary and blood, TSH content is high in the rat and low in the guinea pig. This parallelism does not hold throughout, however. No TSH was detectable in frog serum, yet frog pituitary is allegedly high in thyrotrophin (Adams and Beeman, 1942).

Since the differences in the thyroid-TSH balance of even closely related mammalian forms were striking, it is obvious that a phylogenetic order has not been demonstrated for the vertebrates tested. The results, like those on pituitary thyrotrophin (Adams, 1946), indicate that the content of TSH in blood bears no apparent relation to the evolutionary position of the animal. We consider it likely that the differences in thyroid-TSH balance as found were merely reflections of thyroid and pituitary gland activities for the physiologic requirements existing when blood was drawn, and that in other circumstances the thyroid-TSH equilibrium might be shifted considerably. Individual variations among animals of the same species, however, were not assessed. It is significant that the high titer of TSH in rat blood disappeared after hypophysectomy, and that blood TSH, not demonstrable in normal guinea pigs, became appreciable in animals treated with the anti-thyroid drug. Full significance of the differences in the thyroid-TSH balance in the vertebrate organism must remain obscure until a complete study is made utilizing the same method for blood and pituitary TSH, and in which bioassays are correlated with the state of thyroid gland activity in the donor.

SUMMARY AND CONCLUSIONS

The bioassay of TSH has been re-quantitated in the stasis (starved, non-metamorphosing) tadpole with graded amounts, (1.5–750 μg.) of the International Gland Preparation of desiccated ox anterior pituitary. As little as 7.5 μg. (0.0005JS unit) of the standard powder elevates mean thyroid cell height and increases hindlimbl ength (metamorphosis) in test animals Thyroid activation and hindlimb extension parallel one another, with both responses a linear function of administered TSH. The stasis tadpole is also highly sensitive to thyroid principle. As little as 0.025 μg. of d,l-thyroxine can re-initiate metamorphosis without causing significant change in the resting status of the thyroid gland in the test animal.

Bioassay "recovery" experiments with human sera demonstrate that the stasis tadpole can detect small amounts of thyroid and thy-

rotrophic hormones simultaneously and in the same sample of serum without recourse to chemical or fractionation procedures. Comparative studies, utilizing the technique, indicate that the thyroid-TSH balance in the circulating fluids of vertebrates may vary considerably. In rat serum, TSH concentration is high with little thyroid hormone detectable. Contrariwise, beef, human, lamb, horse, and guinea pig sera have appreciable thyroid but little or no TSH activity. The sera of lower vertebrates (frog, turtle) are ineffective, but pigeon serum has some TSH activity. The thyroid-TSH balance in the blood can be altered experimentally. TSH in rat serum vanishes after hypophysectomy, whereas, thyrotrophin increases to a detectable level in the blood of the goitrous guinea pig.

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ACTION OF INSULIN ON DEPOSITION OF GLYCOGEN AND STORAGE OF FAT IN ADIPOSE TISSUE

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Soon after the discovery of insulin, clinical observers reported the occurrence of atrophies (Depisch, 1926; Barborka, 1926) and hypertrophies (Eeg-Olofsson, 1930; Rowe and Garrison, 1932) of subcutaneous adipose tissue at the site of repeated insulin injections. A number of case reports and statistical studies have since been published. It has been shown that in insulin atrophies the abnormal process consists of simple disappearance of fat without evidence of inflammatory reaction (Marble and Smith, 1942). Moreover, lipodystrophy without evidence of inflammation has not been reported following injection of substances other than insulin. The production of insulin lipodystrophies in animals has been attempted by Reed, Anderson, and Mendel (1930), Marble and Smith (1942), Goldner (1943) and Oesterreicher (1947) with conflicting results.

In the search for an explanation of the effect of insulin on the adipose tissue of diabetics, various factors not concerned with the actual metabolic action of insulin have been suggested but supporting experimental evidence has been lacking (Marble and Smith, 1942). That the lipodystrophies might be due to a local metabolic effect of insulin itself was first suggested by Nichols (1930) but without experimental evidence. Oesterreicher (1947) and Marble and Renold (1949) studied the lipolytic activity of adipose tissue in man and in the rat, and investigated the action of insulin alone and in combination with other hormones on the lipolytic activity of fat, both *in vivo* and *in vitro*. No action of insulin was apparent under the conditions of the experiments.

Arndt (1926) reported that administration of insulin to dogs may result in the deposition of glycogen in adipose tissue. Such an effect of insulin in rats was denied by Richter (1931) employing histological methods, but confirmed by Wertheimer (1945) by chemical

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analyses. In a recent histological investigation of glycogen in adipose tissue Fawcett (1948) showed that glycogen was regularly deposited in the adipose tissues of rats after insulin administration. Alimentary hyperglycemia caused glycogen to be deposited in the fat of normal rats but the fat depots of rats with alloxan diabetes were devoid of histologically demonstrable glycogen. In a few rats of a small experimental group, adipose tissue at the site of insulin injection appeared to contain more glycogen than the fat elsewhere. A possible relation between this observation and the problem of insulin lipodystrophy was suggested.

In the present investigation we have studied the influence of insulin on the deposition of glycogen in the adipose tissues of normal and diabetic rats using quantitative chemical analysis as well as the qualitative histochemical staining method for glycogen. Experiments have been carried out to determine whether insulin does indeed have a local effect on adipose tissue at the site of injection, and if so, whether the activity of the fat cells in this respect is altered by the daily injection of insulin over a period of several weeks. At the same time we observed the sites of daily insulin injection for the development of any form of lipodystrophy.

MATERIALS AND METHODS

Female albino rats weighing 160–220 gm. were used throughout unless otherwise indicated. In view of the clinical finding that insulin lipodystrophies are more frequent in children (Marble and Smith, 1942; Marble and Renold, 1949) young rats weighing 80–120 gm. were used for the long-term experiments. However, at the end of the experiments these animals were within the usual weight range of 160–200 gms. The animals were fed Purina rat chow *ad libitum*.

Protamine zinc insulin (Lilly), diluted with saline just prior to injection so as to contain 10 units per cc., was used unless otherwise stated. Injections were made subcutaneously on the back, or, if the local action of insulin was to be studied, into the groin fat. The groin fat of normal female rats (160–200 gm.) represents a mass of predominately adipose tissue, weighing 0.6 to 0.9 gm. in which however a relatively constant, very small amount of mammary gland tissue and some tiny lymph nodes are embedded. By injection of methylene blue and subsequent dissection the correct injection technique was learned. When the local action of insulin was being studied, insulin was injected into the left groin fat while the same volume of isotonic saline was injected into the right groin fat, which served as a control.

For comparable removal of the groin fat on both sides the following procedure was found satisfactory: after good exposure, the fat was cut through at the inner edge of the adductor muscle mass, then dissected from the underlying muscle until entirely freed both from the leg and the abdominal wall; it was then held up without tension and cut with the scissors lying flat on the leg muscles. In 14 normal animals the maximum difference in weight of the fat removed from the two sides in this manner was 4 per cent. In removing the interscapular brown fat for chemical analysis great care

must be taken to avoid including a portion of the superficial back muscle which runs through each of the symmetrical brown fat pads.

The glycogen determinations in adipose tissue were made by the method of Good, Kramer and Somogyi (1933). Inasmuch as the amounts of glycogen were small, except in the brown fat, a number of recovery experiments for small amounts of glycogen added to adipose tissue were made (see Table 1) to determine the accuracy of the method at these levels. To control individ-

TABLE 1. RECOVERY OF SMALL AMOUNTS OF GLYCOGEN ADDED TO ADIPOSE TISSUE OF THE RAT

Adipose tissue mg.	Glycogen added mg.	Glycogen found mg.	Recovery per cent
910	0.00	0.00	—
620	0.31	0.35	113.0
640	0.62	0.60	96.7
850	0.93	0.93	100.0
1050	1.25	1.12	90.0
875	1.87	1.65	88.0
1025	2.50	2.38	95.0

ual variation, on the other hand, the glycogen in the fat of both groins was determined and compared in a number of previously injected animals (see Table 2). For amounts of glycogen in fat ranging from 10 to 300 mg. per 100 gm. the average difference between the two groins of the same animal was 0.9 mg. per 100 gm. and the standard error 4.4 mg. per 100 gm. or, if expressed in per cent difference, $1\% \pm 2.9\%$.

Total ether extractable material was determined by mincing the adipose tissue in a Waring Blendor with a large volume of ether; the ether was filtered, evaporated, and the residue re-extracted with a small volume of ether, evaporated and brought to constant weight.

TABLE 2. GLYCOGEN IN GROIN FAT PADS OF 6 NORMAL AND 6 DIABETIC RATS, 24 HOURS AFTER THE INJECTION OF 8 UNITS OF PROTAMINE ZINC INSULIN AT A DISTANT SITE

Subjects	Groin fat, weight in mg.		Glycogen in groin fat, mg. per cent		Difference in glycogen Left > Right	
	Right	Left	Right	Left	Mg. per cent	Per cent
Normal rats	875	880	31	35	+ 4	+12
	860	865	10	10	0	0
	1380	1430	30	28	− 2	− 7
	870	825	18	15	− 3	−16
	860	865	15	18	+ 3	+20
	875	880	21	22	+ 1	+ 5
Diabetic rats	600	600	66	66	0	0
	490	480	160	159	− 1	− 0.5
	510	575	73	70	− 3	− 4
	625	500	190	160	−30	−16
	500	460	250	290	+40	+16
	545	620	120	122	+ 2	+ 2
Average difference					0.9 ± 4.4*	1 ± 2.9*

* Standard errors.

Rats were made diabetic by the subcutaneous injection of 180 mg./kg. alloxan monohydrate (Eastman Kodak Co.) after a 24 hour fast. On the second and seventh days after injection, the presence of diabetes was demonstrated by glucose determinations on blood obtained from the tail of the non-fasted animal. The micromethod of Folin and Malmros (1929) was used and levels of 200 mg. per 100 cc. were considered indicative of diabetes. If the animals were kept for a longer period of time, the persistence of hyperglycemia was always determined before use.

For histological study, tissue was fixed in Rossman's fluid and stained by the periodic acid-Schiff method of McManus (1946). For the study of the general topography of the tissue and the size of the fat cells parallel sections were stained by Masson's trichrome method.

RESULTS

General Effect of Insulin on Adipose Tissue. The effect of insulin on glycogen deposition in the fat of both normal and diabetic rats was studied by histological and chemical methods. Particular attention, however, was given to the action of insulin on the fat of *diabetic rats* since this was not included in previous studies (Fawcett, 1948). The absence of histologically demonstrable glycogen in the adipose tissue of untreated diabetic rats was confirmed. Three and 6 hours after the administration of 5 units of crystalline insulin (Lilly) to diabetic rats, histological sections revealed glycogen in the white fat of the groin and in the interscapular brown fat. Glycogen was much more abundant in the brown than in the white fat. When 5 units of protamine zinc insulin were injected, histological sections of the fat were positive for glycogen at 6 hours and strongly positive at 13 hours. The same dosage of insulin appeared to result in deposition of more glycogen in diabetic rats than it did in the fat of normal rats. This histological impression was borne out by chemical analyses (see Table 3).

TABLE 3. ACTION OF INSULIN ON GLYCOGEN DEPOSITION IN ADIPOSE TISSUE

Group of rats	No. of rats	Glycogen in adipose tissue. Average values, mg. per 100 gm.		
		Right groin	Left groin	Interscapular
Normal	6	<5†	<5	48 ± 16.5*
Diabetic	6	<5	<5	34 ± 11.7*
Normal 24 hours after 8 units protamine zinc insulin	6	21 ± 3.4*	21 ± 3.3*	700 ± 291*
Diabetic 24 hours after 8 units protamine zinc insulin	6	143 ± 29.2*	145 ± 34.1*	3300 ± 708*
Diabetic 20 hours after 5 units protamine zinc insulin injected into *left groin*	6	38 ± 10.2*	112 ± 22.8	1400 ± 411*

* Standard errors.
† Lower limit of sensitivity of the method.

It will be noted that although the white fat of both normal and untreated diabetic rats contained no glycogen by chemical analysis, some glycogen was found in the interscapular brown fat of these animals. Glycogen may well occur in brown fat in very small amounts. However, owing to the intimate association of muscle with the interscapular fat bodies, the practically unavoidable inclusion of minute fragments of muscle cannot be excluded.

It must be emphasized that the amounts of glycogen shown in the table represent per cent of fresh weight of tissue. In the cells of ordinary white adipose tissue the volume of the protoplasm represents only 2.4 per cent of the total volume of the fat cell, the remainder being occupied by stored neutral fat (Gersh and Still, 1945). If this be borne in mind, the glycogen values in proportion to volume of active protoplasm are still more striking.

TABLE 4. LOCAL ACTION OF INSULIN IN 6 DIABETIC RATS

5 units protamine zinc insulin into fat pad of left groin control. Same volume saline into fat pad of right groin. Glycogen determinations 20 hours later.

Glycogen in mg. per 100 gm.			Glycogen
Right groin	Left groin	Increase Left > Right	Per cent increase Left > Right
16	65	49	306
61	150	89	146
51	140	89	175
10	27	17	170
52	120	68	131
51	170	119	233
Average increase Left > Right		72 ± 15*	194 ± 27.1*

* Standard errors.

Local Effect of Insulin on Adipose Tissue. It was thought important to see whether or not a *local* action of insulin on the deposition of glycogen in white fat could be verified. Chemical analysis, permitting quantitative comparison, was regarded as the method of choice. A number of preliminary trials were made using various doses of crystalline and protamine zinc insulin and with different time intervals between the injection and the glycogen determination. The best results were obtained by carefully injecting 5 units of protamine zinc insulin, diluted to 0.5 cc. with isotonic saline, into as wide an area of groin fat as possible. The tissues for glycogen determinations were excised 20 hours after the injections. Six diabetic rats were treated in this way and the results are shown in Table 4. Although the actual amounts of glycogen are small, the control experiments (Tables 1 and 2) as well as the statistical analysis clearly show that significantly greater amounts of glycogen are found in the groin fat injected with insulin than in the groin fat injected with isotonic saline alone.

The Effect of Prolonged Daily Injections of Protamine Zinc Insulin

into the Adipose Tissue of Rats. Both normal and diabetic rats were used in this experiment. Five units of protamine zinc insulin made up to 0.5 cc. of isotonic saline were injected daily into the left groin fat, while 0.5 cc. of isotonic saline alone was injected into the right groin fat. This dosage of insulin approached the maximum which could be tolerated daily by the animals used, since at the beginning of the experiment several animals were lost in hypoglycemia. Two blood sugar values of 22 and 26 mg. per cent were recorded. The animals were then offered in addition to their drinking water, a 15 per cent solution of sucrose of which they drank an estimated 5 cc. daily. After this no hypoglycemic deaths occurred but blood sugar values taken from time to time on various normal and diabetic rats were usually below 80 mg. per 100 cc.

TABLE 5. ACTION OF PROLONGED INJECTION OF PROTAMINE ZINC INSULIN ON WEIGHT AND FAT CONTENT OF RAT ADIPOSE TISSUE

Daily injections of 5 units protamine zinc insulin into left groin fat. Control injections of same volume of saline into right groin fat.

A. *Action on weight*

	No. of rats	Days of injection	Average weight of groin fat pads		Average increase Left > Right per cent	Range of increase Left > Right per cent
			Right mg.	Left mg.		
Normal	14	0	784	792	1	− 3 to + 5
	7	30–74	1328	1826	36	+22 to + 62
Diabetic	27	34–66	676	1047	55	+18 to +118

B. *Action on total ether extractable material (EEM)*

			Average EEM of groin fat pads			
Normal	6	0	429	426	− 1	− 2 to + 3
	8	30–74	729	882	21	+10 to + 51
Diabetic	7	38–66	265	440	66	+51 to +100

After 60 days all of the animals were killed and the weights of the groin fat of both sides were recorded. Three groups were formed: In the first group, total ether extractable lipids were determined in the fat of both groins; in the second group the fat of the two sides was studied histologically; and in the third group glycogen determinations were done chemically. The results in all animals which lived beyond the thirtieth day of insulin and saline injections are summarized in tables 5 and 6. It should be emphasized that, with 2 exceptions, the animals were sacrificed 48 hours or more after the last injections.

It is apparent from a study of the tables that in all 34 animals, both normal and diabetic, *the fresh weight* of the insulin-injected rat (left) was considerably greater than that of the saline-injected (right) side. The increase in weight of the insulin-injected side occurred in every animal in the series and averaged 36 per cent for the 7 injected

normals and 55 per cent for the 27 diabetic rats. In some diabetic rats the increase of left over right was as great as 118 per cent, while in the 14 control animals this weight difference, left over right, varied only between −3 and +5 per cent.

That this increase was due, at least in part, to actual increase in fat and not to edema, hemorrhage or inflammatory reaction is shown (1) by the increase in *total extractable lipids* averaging 21 per cent in the 8 normal rats and 66 per cent in the 7 diabetic animals examined, as compared with −2 to +3 per cent in the 6 controls and (2) by the histological examination in which only 3 of 14 animals showed a sig-

TABLE 6. DEPOSITION OF GLYCOGEN IN ADIPOSE TISSUE 24 HOURS AFTER 8 UNITS OF PROTAMINE ZINC INSULIN, IN DIABETIC RATS SUBJECTED UNTIL 5 DAYS PREVIOUSLY AND FOR 60–64 DAYS TO DAILY INJECTIONS OF 5 UNITS OF PROTAMINE ZINC INSULIN INTO THE LEFT GROIN FAT

Weight of fat pads			Glycogen found					
			Actual amounts			Percentage		
R. groin mg.	L. groin mg.	Interscapular (brown) fat mg.	R. groin mg.	L. groin mg.	Interscapular (brown) fat mg.	R. groin mg. per cent	L. groin mg. per cent	Interscapular (brown) fat mg. per cent
450	550	650	2.07	1.5	18.75	380	270	2900
675	925	375	1.6	1.65	14.2	250	180	3800
840	1040	450	1.25	1.43	8.3	150	140	1800
860	1325	560	2.25	2.62	8.75	260	200	1550
550	800	700	1.6	1.65	14.0	290	200	2000
800	1080	600	2.2	2.25	20.0	270	210	3330
Averages 696	953	556	1.83	1.85	14.0	263	196	2518

nificant amount of fibrosis or inflammatory reaction. Furthermore in the majority of animals the average size of the individual fat cells appeared quite definitely increased in the insulin-injected groin. This is illustrated in Plate 1.

Histological examination of fat from two diabetic rats 24 hours after the last of 66 daily injections of insulin, and from two diabetic rats 48 hours after the last injection, disclosed no glycogen either in the injected or in the control groin, nor in the interscapular brown fat. Hence, although the deposition of conspicuous amounts of glycogen in the adipose tissue of diabetic rats could be demonstrated 24 hours after a single injection of 5 units of protamine zinc insulin, this was not the case twenty-four hours after the last of 66 daily injections of insulin in the same dosage. An attempt was therefore made to determine whether the ability of the injected adipose tissue to store glycogen had been altered by the repeated daily injections. The diabetes of 8 such animals was allowed to remain uncontrolled for 5 days (the persistence of diabetes was verified by blood sugar determinations on the third day) and on the sixth day 6 units of protamine zinc insulin were injected at a distant site. The fatty tissues were excised 24 hours later for chemical glycogen determinations. The results are

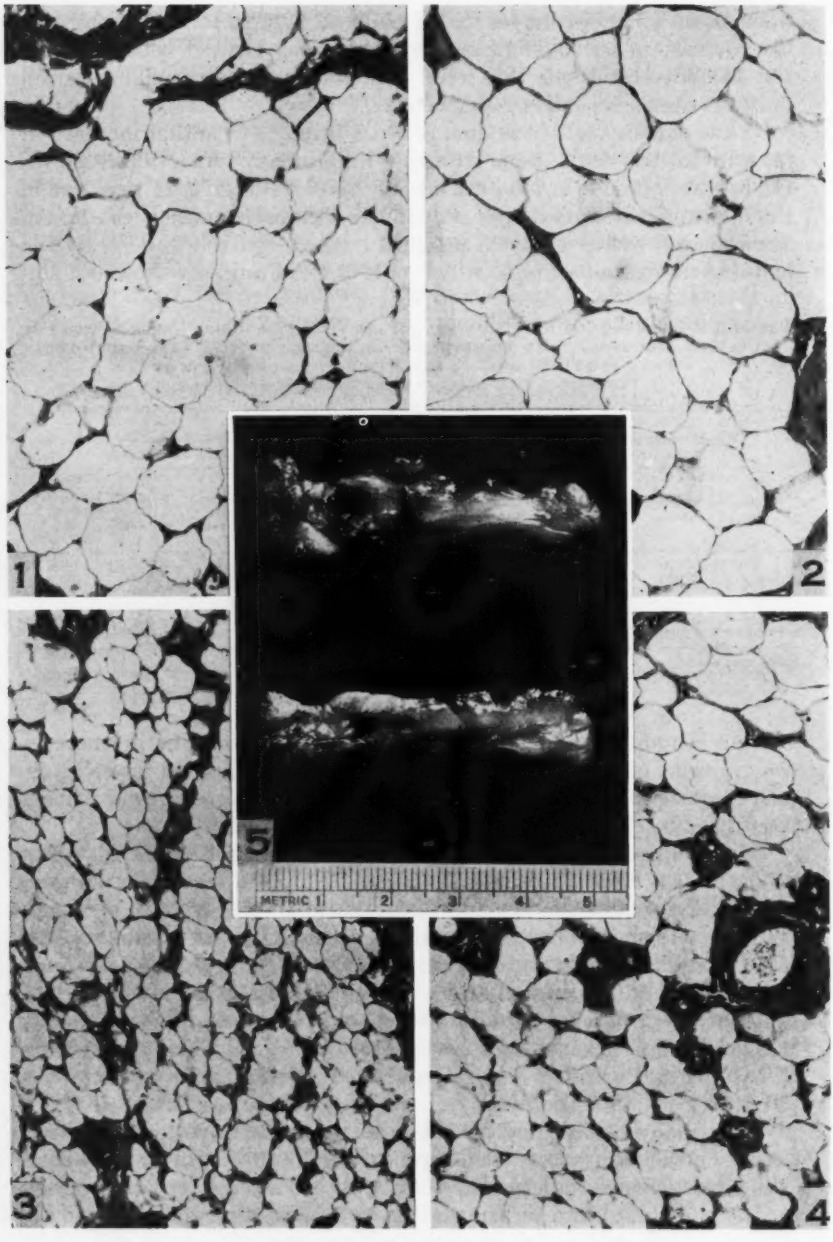

PLATE 1

given in Table 6. The amounts of glycogen deposited were comparable with those found after insulin injection in untreated diabetic rats and were the same on both sides. It is worth pointing out that the "hypertrophied" insulin-injected side did not deposit more glycogen than the control side; this can be interpreted as further evidence for an increase in stored fat rather than in number of fat cells. The amount of cytoplasm of fat cells presumably remains the same and hence about the same amount of glycogen is deposited.

DISCUSSION

In addition to what is known of the systemic, general action of insulin on adipose tissue, as shown by Arndt (1926), Wertheimer (1945) and Fawcett (1948), the data presented suggest two *direct, local* actions of insulin on subcutaneous fat:

(1) The deposition of glycogen at the site of a single injection.

(2) A significant hypertrophy of the fatty tissue at the site of repeated injections. This hypertrophy represents at least in part an actual increase in lipids, and there is evidence to indicate that this is due to an increased amount of fat stored in the individual fat cells.

The experimental work concerning the metabolic function of the cells of adipose tissue has recently been reviewed by Fawcett (1948) and Wertheimer (1948). This may be summarized as follows: Whereas the opinion had long been held that adipose tissue was a metabolically inert storage tissue and that most important steps of fat metabolism occur in the liver alone, the use of isotopes in recent years has shown that assumption to be incorrect. Schoenheimer and his coworkers (1942) demonstrated that the lipids in adipose tissue are in a constant state of synthesis and degradation, and Shapiro and Wertheimer (1948) gave conclusive evidence, *in vitro*, for the synthesis of fatty acids in isolated adipose tissue itself.

Schoenheimer and Rittenberg (1936) and Bernhard and Schoenheimer (1940) established the existence of synthesis of fatty acids from carbohydrates in the body. Stetten and Boxer (1944 a) showed that 30 per cent of ingested carbohydrate is stored as fat in the rat. This synthesis of fatty acids from carbohydrates is activated by insulin, and is reduced to 5 per cent of normal in the diabetic rat (Stetten and Boxer, 1944 b). The seat of this conversion again is

FIGS. 1 and 2. Two representative areas from sections of fat from the left groin of a diabetic animal (#59) after 66 daily injections of 5 units of protamine zinc insulin into the left groin. Note the size of the fat cells. Masson's trichrome stain. ×180.

FIGS. 3 and 4. Two representative areas from sections of fat from the right groin of the same animal as Figs. 1 and 2. The right groin served as control and was injected daily for 66 days with isotonic saline alone. The largest cells seen in the section are those in Fig. 4. Masson's trichrome stain. ×180.

FIG. 5. Fat mass of both groins of the same animal as Figs. 1–4 excised in comparable manner as described under "methods." Above: Fat of the left groin insulin-injected for 66 days. Below: Fat of the right groin saline-injected. Note the hypertrophy of the left groin fat.

generally believed to be the liver. Tepperman, Brobeck and Long (1943) however demonstrated that the increased fat synthesis produced in rats by dietary training persisted even in the absence of the liver, and that this extrahepatic mechanism of fat synthesis was activated by insulin.

The glycogen deposition in adipose tissue is a further evidence of its metabolic activity. The conditions under which glycogen is deposited are generally the same as those enhancing the deposition of fat (glycogen deposition precedes fat deposition and comes to an end with completion of the latter). Furthermore the respiratory quotient of adipose tissue containing glycogen is *in vitro* greater than 1 (Ruska and Quast, 1935 and Mirsky, 1942) as compared to 0.7 if no glycogen is present. Finally, in diabetic animals, in which the conversion of carbohydrate to fat is greatly reduced, no glycogen is deposited under conditions which normally lead to the deposition of conspicuous amounts.

There is considerable evidence therefore suggesting not only that at least part of the transformation of carbohydrate to depot fat is extrahepatic but also that it may well take place in the fatty cell itself. This hypothesis was first formulated by von Gierke in 1906.

We believe the experimental data here reported lend further support to this hypothesis. Three mechanisms could account for the increased fat deposition under the local action of insulin: decreased fat mobilization, increased storage of fat synthesized elsewhere (liver) or increased synthesis of fat locally. As far as we are aware none of the known effects of insulin would account for the first two mechanisms suggested. Locally increased fat synthesis from carbohydrate, however, could be related to the deposition under these circumstances of metabolically active carbohydrate (glycogen) in the fat cells and to the known effect of insulin on the conversion of carbohydrate to fat in the body (Stetten and Klein, 1946). Furthermore in animals with uncontrolled diabetes of several days' duration and therefore depleted fat stores, the same dose of insulin produces a significantly greater glycogen deposition than in the non-diabetic animals (see Table 3).

It is noteworthy that the action of insulin on adipose tissue leading to hypertrophy is not only a local but also a systemic one. In the non-diabetic rats receiving daily injections of insulin the increase in subcutaneous fat, as compared to the non-diabetic animals receiving no insulin, was apparent not only in the insulin-injected, hypertrophied groin fat but also on the control side (Table 5 A and B). This lends support to the view that the action of insulin on adipose tissue is a true metabolic action of insulin itself, and is not due to any factor incidental to the injection such as trauma, foreign proteins, pH, contaminants, preservatives in commercial insulin or other influences.

The aim of this study originally was to elucidate the pathogenesis of insulin lipodystrophies. In this respect no final conclusion could be reached. However, the data strongly suggest that insulin has a direct metabolic effect on adipose tissue, and that this results primarily in local hypertrophy. Fawcett (1948) had suggested that the atrophic type of lipodystrophy might contrarily result from a breakdown of the fat cell metabolism following prolonged and possibly excessive stimulation. In our experiments however, the ability of the fat cells to store glycogen after the injection of insulin was not altered locally by 66 daily insulin injections. In the rat the atrophic type of lipodystrophy could not be experimentally reproduced and the pathogenesis of this condition in diabetic patients is as yet unexplained.

SUMMARY

The metabolic effects of insulin on adipose tissue have been investigated, with particular attention to its local action on fat cells at the site of injection.

After administration of a large dose of either crystalline or protamine zinc insulin the deposition of glycogen in fat cells is significantly greater in diabetic rats than in normal animals. Moreover, a greater amount of glycogen is deposited at the site of injection than elsewhere.

After the daily injection of large doses of protamine zinc insulin for 5 to 9 weeks into the fat of one groin of normal and diabetic rats, with control injections of isotonic salt solution into the other groin, hypertrophy of the insulin-injected adipose tissue was demonstrated. This was evidenced by an increase in weight averaging 55 per cent in the 27 diabetic animals and an increase in total lipids averaging 66 per cent of the values obtained on the saline-injected, control side. Histological examination revealed an increase in the volume of the individual fat cells of the insulin-injected fat.

It is concluded that insulin indeed has a direct, local action on the adipose tissue at the site of injection. There is evidence to suggest that this may be due to a promoting action on the synthesis of fat from carbohydrate in the fat cell itself.

The relation of these findings to the clinical occurrence of insulin lipodystrophies is discussed.

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THE EFFECT OF A STRESS UPON THE GLYCOSURIA OF PARTIALLY DEPANCREATIZED FORCE-FED RATS

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KALAMAZOO, MICHIGAN

THE HYPOTHESIS that any severe stress should intensify diabetes by activating the adrenal cortices is based upon the following observations: 1. The secretory activity of the adrenal cortices is increased during most if not all types of stress (Sayers and Sayers, 1948); 2. Adrenal cortex extracts and steroids are diabetogenic (Long, Katzin and Fry, 1940) and 3. Activation of the adrenal cortices by the administration of adrenocorticotrophic hormone intensifies diabetes (Ingle, Prestrud and Li, 1948).

In the present study, the injection of dilute solutions of formaldehyde was used as a stress to partially depancreatized, force-fed rats. In moderately diabetic rats, the administration of formaldehyde was followed by some decrease in the levels of glycosuria in all of the animals. Injections of formaldehyde did not affect the level of glycosuria in severely diabetic rats.

METHODS

Male rats of the Sprague-Dawley strain of approximately 300 gm. were subjected to partial pancreatectomy by the technique of Ingle and Griffith (1942). In most of the animals, the pancreas lying between the bile duct and the duodenum was left intact. These animals exhibited "moderate" diabetes. For the production of "severe" diabetes, almost all of the pancreas was removed and only tiny remnants remained in the area of the bile duct. Following recovery from operation, the animals were placed in metabolism cages and fed a medium carbohydrate diet. All of the animals were force-fed by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The techniques and diet were modifications of those described by Reinecke, Ball and Samuels (1939). The experiments were carried out in an air-conditioned room at a temperature of 74 to 78 degrees F. and humidity at 30 to 35 per cent of saturation. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with thymol. Urine glucose was determined by the method of Benedict (1911).

A solution of 1.5 per cent formaldehyde was given by subcutaneous injection twice each day following each feeding for periods of 7 or for 28 days.

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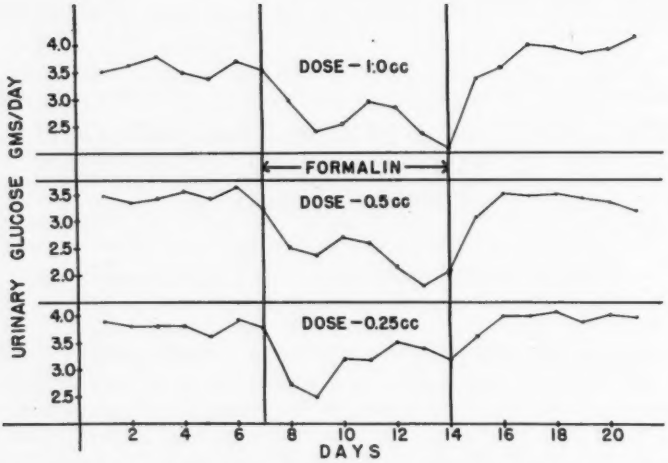

FIG. 1. Urinary glucose in partially depancreatized, moderately diabetic rats. Averages based upon 5 rats per group.

EXPERIMENTS AND RESULTS

In experiment 1 (Fig. 1) 15 moderately diabetic rats were observed during a control period of 14 days. Five rats were given 0.25 cc. of formaldehyde solution twice daily for 7 days; five rats were given 0.5 cc. twice daily for 7 days, and 5 rats were given 1.0 cc. twice daily for 7 days. All of the animals in each group showed a decrease in the level of urinary glucose during the administration of formalde-

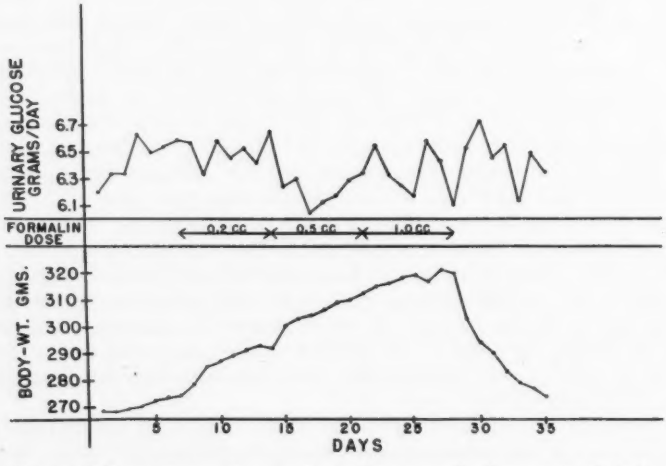

FIG. 2. Urinary glucose in extensively depancreatized, severely diabetic rats. Averages based upon 5 rats.

hyde and a return to the preinjection level of glycosuria following the termination of injections.

In the experiment 2 (Fig. 2), 5 severely diabetic rats were given 0.2 cc. of formaldehyde solution for 7 days, 0.5 cc. for 7 days, and 1.0 cc. for 7 days. There was no significant change in the level of urinary glucose during any phase of the experiment. The data on weight (Fig. 2) illustrate the gains in weight caused by the development of edema at the site of injection. When the injections of the formaldehyde solution were stopped, the body-weight rapidly returned to the preinjection level.

In experiment 3 (Fig. 3), 5 moderately diabetic rats were observed during a control period of 14 days. All of the rats were given 0.25 cc. of formaldehyde solution twice daily for 28 days and were observed

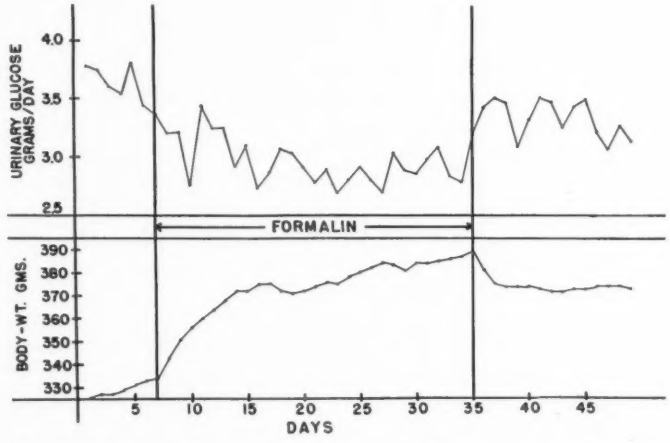

FIG. 3. Urinary glucose in partially depancreatized, moderately diabetic rats. Averages based upon 5 rats.

for 14 days following the termination of injections. All of the animals showed a decrease in the level of urinary glucose during the administration of formaldehyde and a return to the preinjection level of glycosuria following the termination of injections.

The injections were tolerated by the rats with little evidence of pain, but some destruction of the subcutaneous tissues occurred and doses of 0.5 and 1.0 cc. caused the animals to appear weak and ill during the first few days. No diarrhea developed in any of the animals and intestinal absorption appeared to be unaltered by the injections.

DISCUSSION

These data support our few earlier observations (Ingle, 1941; Ingle and Nezamis, 1943) on the effect of stress in the partially de-

pancreatized rat. The injection of solutions of formaldehyde represents a severe stress, and there is every reason to believe that the adrenal cortex is stimulated to increased secretory activity under these conditions, (Selye, 1937; Sayers and Sayers, 1948). It would seem that whereas activation of the adrenal cortex by the administration of adrenocorticotrophic hormone under non-stress conditions causes intensification of glycosuria, activation of the adrenal cortex by stress may fail to cause increased glycosuria and is even compatible with some decrease in glycosuria. In discussion of this apparent paradox, Ingle (1948) has suggested that an increase in the activity of the adrenal cortices in the absence of an increased "need" for the extra hormone does cause over-dosage phenomena, but that the same output of hormone during increased "need," as during stress, tends to sustain homeostasis rather than to cause overdosage phenomena. It is possible to favor this hypothesis that changes in the secretory activity of the adrenal cortex normally support homeostasis and still recognize the possibility that dysfunctions of the adrenal cortex, representing imbalances in the output of the cortical hormones, may arise during disease or other forms of stress and cause other pathologic changes. Selye (1948) has suggested that the increased production of corticoids may cause hyperglycemia during the phase of resistance to a stress. In these experiments, the animals given 0.25 cc. of formaldehyde solution twice daily for 28 days were regarded by us as being in the stage of resistance before the termination of injections. There was no tendency toward elevation of the level of urinary glucose before the injections were stopped.

By what mechanism does the injection of solutions of formaldehyde cause a decrease in glycosuria in the mildly diabetic rat? We are unable to answer this question, but it seems necessary to assume that this form of stress accelerates some phase of carbohydrate utilization or inhibits its formation in the rat having a mild insulin deficiency. There was no demonstrable effect in the severely diabetic rat. Ingle and Nezamis (1948) reported that the stimulation of muscle increases the glucose tolerance of insulin-deficient eviscerate rats.

When it became clearly established (Long and Lukens, 1936) that adrenalectomy causes a significant amelioration of experimental diabetes, the question was frequently raised as to whether any other debilitating condition would do the same. The finding that an excess of the cortical hormones intensified diabetes and even caused glycosuria in normal animals (Ingle, 1941) has been considered proof that the changes in carbohydrate metabolism during adrenal cortical insufficiency and excess represents some primary function of the adrenal cortex. In the opinion of the writer, the effect of other natural products, drugs and physiological abnormalities upon diabetes has not been adequately studied under conditions of controlled caloric intake.

SUMMARY

Partially depancreatized male rats were force fed a fluid diet. The subcutaneous injection of solutions of 1.5 per cent formaldehyde in doses of 0.25, 0.5, and 1.0 cc. twice daily for 7 days, 5 rats per dose level, caused some decrease in the level of urinary glucose in mildly diabetic rats. When the injections were stopped, the glycosuria was reestablished at its preinjection level. The administration of similar amounts of formaldehyde to 5 severely diabetic rats did not affect the level of glycosuria. Five moderately diabetic rats were given 0.25 cc. of 1.5 per cent formaldehyde solution twice daily for 28 days. There was a decrease in the level of urinary glucose in each animal which persisted for the duration of the injections. These data do not support the hypothesis that activation of the adrenal cortices during stress causes exacerbation of diabetes.

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HISTOPATHOLOGICAL CHANGES INDUCED IN THE NORMAL THYROID AND OTHER TISSUES OF THE RAT BY INTERNAL RADIATION WITH VARIOUS DOSES OF RADIOACTIVE IODINE[1]

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PREVIOUS investigations dealing with the histopathological changes induced by radiation in the *normal* thyroid gland may be grouped according to whether the radiation was applied externally by X-ray or internally by either the implantation of radium needles or the administration of radioactive iodine. The early studies dealing with X-ray and radium needle implants, which have been reviewed by Warren (1943), have served to emphasize the resistance of the normal thyroid gland to radiation.

Destruction of variable amounts of the normal thyroid gland by the administration of radioactive iodine has been reported: in the dog, by Hamilton and Lawrence (1942) and by Hamilton (1942); in the rabbit, by Hamilton and Lawrence (1942); in the adult rat, by Findlay and Leblond (1948); in the new-born rat, by Goldberg and Chaikoff (1949); and in the mouse, by Gorbman (1947, 1949).

The purpose of the present investigation was to provide, in some detail, a continuous picture of the progressive changes induced in the thyroid gland by internal, ionizing radiation. The reactions to radiation injury were followed for frequent intervals up to three days, then weekly up to one month, and thereafter at five, six, and eight months following *single* injections of 18, 300, 525, and 875 microcuries of I^{131}.[4] In the course of this study, information has been obtained on (1) the dose required for complete destruction of the thyroid gland of the adult rat; (2) a dose which destroys a large part of the gland but does not prevent complete regeneration; (3) the dose of I^{131} which, because it produces no observable histological damage, can be safely used as a tracer for metabolic studies.

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[2] Atomic Energy Commission Fellow.

[3] Public Health Research Fellow of the National Cancer Institute.

[4] The microcurie as used here is based on Bureau of Standards recalibration of the I^{131} millicurie in effect at Oak Ridge National Laboratories July 1, 1949 (cf. Isotopes Division Circular E-44 from U. S. Atomic Energy Commission).

EXPERIMENTAL

Male rats of the Long-Evans strain, weighing in the neighborhood of 250 gm. each, were used throughout. They were maintained on a diet (No. 1) the composition of which has been described elsewhere and which contained about 0.3 microgram of I^{127} per gram (Taurog and Chaikoff, 1946). The rats were permitted access to food and water throughout the entire periods of observation.

Each rat received a single, intraperitoneal injection of either 18, 300, 525, or 875 microcuries of I^{131} to *which no carrier had been added.* The rats were kept in metal cages with screen bottoms to prevent access to feces. At various intervals after the I^{131} injection, the rats were anesthetized with sodium pentobarbital (one cc. of two per cent solution per rat), and exsanguinated.

TABLE 1. EXPERIMENTAL ARRANGEMENT AND DOSAGES OF I^{131} INJECTED

I^{131}-injected	Interval sacrificed after injection	Number of rats in each group	Dose delivered to a point at the center of the thyroid gland
microcuries			rep*
Controls	6 hrs–8 mo.	36	
875	6 hours	8	
875	12 hours	8	66,000
875	24 hours	12	160,000
875	48 hours	8	
875	72 hours	8	330,000
875	1 week	12	
875	2 weeks	4	
875	3 weeks	4	
875	4 weeks	8	
875	6 months	6	
875	8 months	6	
525	6 hours	4	
525	12 hours	4	
525	24 hours	4	
525	48 hours	4	
525	72 hours	4	
525	1 week	4	
525	1 month	4	
525	5 months	2	
525	6 months	4	
525	8 months	12	
300	6 hours	8	
300	12 hours	8	19,000
300	24 hours	8	53,000
300	48 hours	8	
300	72 hours	8	110,000
300	1 week	8	
300	4 months	6	
300	8 months	8	
18	6 hours	4	
18	12 hours	4	820
18	24 hours	4	2,400
18	72 hours	4	7,900
18	216 hours	4	12,000
18	1 month	4	

* rep = roentgens equivalent physical.

The soft tissues of the neck anterior to the prevertebral fascia were removed *in toto* and fixed in Bouin's fluid for histological examination. Representative portions of the gastrointestinal tract, kidney, genito-urinary tract, liver, and heart were treated in a similar manner. Serial sections, 6–10 microns thick, were made of the neck region. Other tissues were sectioned at six microns. The sections were stained with hematoxylin and eosin.

Groups of rats (four rats per group) were sacrificed at various intervals after the I^{131} injections for determination of the radioactivity present in the thyroid glands. Approximately one half of one lobe of each gland was used for histological examination. Radioactive measurements were carried out on the remaining thyroid tissue by the method described in an earlier communication (Feller *et al.*, 1949).

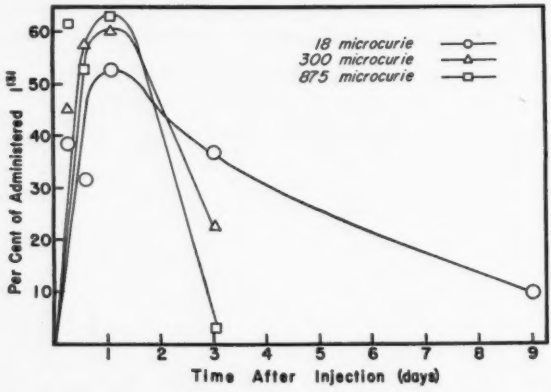

FIG. 1. The per cent uptake and retention of a single injection of 18, 300 and 875 microcuries of carrier-free radioactive iodine by the rat thyroid gland. The more rapid loss of the trapped radioiodine with the higher doses reflects the observed glandular destruction. This should be contrasted with the retention of the 18 microcurie dose, one which resulted in no observable damage to the thyroid gland.

RESULTS

The data presented below are based on the examination of the tissues of over 250 rats. Groups, as shown in Table 1, were injected with either 18, 300, 525, or 875 microcuries of carrier-free I^{131}. The uptake-retention curves of the injected I^{131} for all but the 525 microcurie dose are shown in fig. 1. The dose, delivered to a point at the center of the gland at the intervals studied, has been estimated by the method described by Feller *et al.* (1949). The values expressed in roentgen-equivalent physical (rep) are recorded in Table 1.

I. *Rats Injected with 875 Microcuries of I^{131}*

Reactions Observed During the First Day (Figs. 2–4).—No histological changes in the thyroid gland were detected six hours after the

single injection of this dose, but by the time 12 hours had elapsed, swelling and vacuolation of the cytoplasm of many epithelial cells were prominent (fig. 2). In the thyroid of one rat, additional degenerative changes were already visible in the nuclei. There was folding and wrinkling of the nuclear membrane, and the chromatin material was condensed and deeply stained.

Twenty-four hours after the injection, the thyroids showed both capsular and interstitial edema, the latter resulting in wide separation of the follicles (figs. 3 and 4). The glands were infiltrated with lymphocytes, macrophages, fibrin, and many neutrophilic leucocytes (fig. 4). Some follicles contained extensively vacuolated colloid. Their epithelial cells were swollen and vacuolated. There was evidence of follicular disintegration, nuclear pyknosis, and extensive epithelial desquamation into the follicular lumens. The first reaction in the arterioles and capillaries was noted at this time, and consisted of bulging of the swollen endothelial cells into the vascular lumens.

Reactions Observed at 48 Hours (*Figs. 5–7*).—At this time, there was massive destruction of the thyroid glands (figs. 5 and 6). The few recognizable follicles that remained were lined by extremely swollen vacuolated cells (fig. 7), obviously in a late stage of degeneration. Some of these follicles still contained dark-staining colloid. Aside from the few remaining follicles, the gland was characterized by severe edema with infiltration of lymphocytes, neutrophilic leucocytes, macrophages, and considerable deposition of fibrinous material. The blood vessels were also involved, and showed pronounced edema of their walls and desquamation of the endothelial cells. No distinct perivascular infiltration was noted. The collagenous tissue adjacent the thyroid appeared fibrillar and granular.

Reactions Observed at 72 Hours (*Figs. 8–10*).—Disorganization of the gland was almost complete at this time (fig. 8). The follicles were recognizable only by their stromal framework. A few thyroid cells were still present, but the majority lacked intact nuclei (fig. 8). The glands were devoid of colloid, although a few of these follicular "ghosts" contained granular and fibrillar eosinophilic material that more closely resembled fibrin than colloid (fig. 9).

The fibrinous infiltration, the edema, and the intense cellular reaction in the gland were present to a degree more advanced than that seen at 48 hours. In addition, lymphocytes, macrophages, neutrophilic leucocytes, and fibrin infiltrated the adjacent adipose and muscular tissue (fig. 10). Many of the fibers of the adjacent edematous muscle had lost their striation, and the sarcoplasm appeared homogeneous. Endothelial desquamation and edema of the walls of the arteries were still prominent and, in addition, thrombi were observed for the first time. No pathological changes were detected in the recurrent laryngeal nerves studied with the ordinary hematoxylin-eosin preparations.

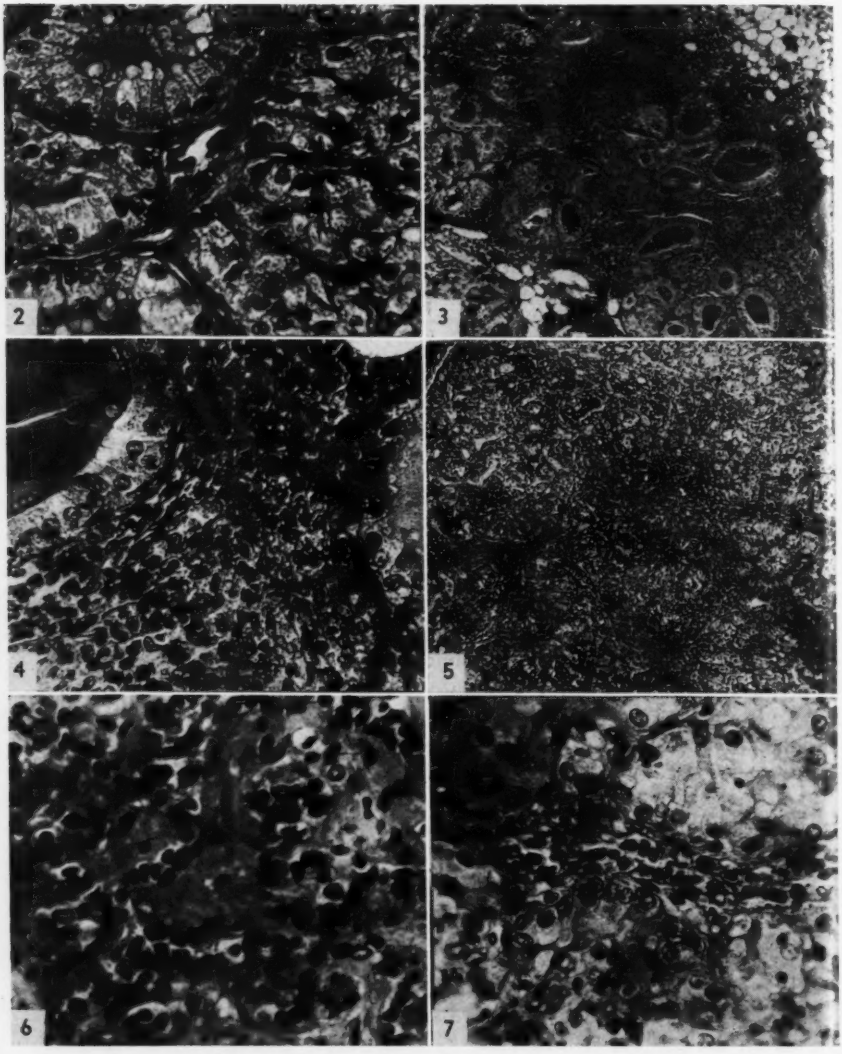

FIG. 2. Thyroid gland of rat sacrificed 12 hours after the injection of 875 microcuries of I^{131}. Note the vacuolation and swelling of the epithelial cells. Hematoxylin and eosin; ×400.

This gland should be compared with an apparently normal gland shown in fig. 28.

FIG. 3. Thyroid gland from rat sacrificed 24 hours after the injection of 875 microcuries of I^{131}. The pronounced edema and intense inflammatory reaction, causing wide separation of the follicles, is observed at this early time. Note the tall, columnar, epithelial cells. Hematoxylin and eosin; ×80.

FIG. 4. Higher magnification of portion of gland shown in Fig. 2. The lymphocytic and neutrophilic character of the infiltrating cells can be seen. Hematoxylin and eosin; ×400.

A few pyknotic epithelial cells were noted in the peripheral regions of the parathyroid glands.

Reactions Observed at the End of One Week (Figs. 11–12).—The outlines of the follicles in the thyroid gland were still visible, and these structures were distended with fibrin and red blood cells (fig. 11). Only a few scattered thyroid epithelial cells were present, and these were found mainly in the periphery of the gland. These peripheral, unfragmented epithelial cells contained little cytoplasm, and the majority were in various stages of degeneration and necrosis. The infiltration of neutrophilic leucocytes, though mild in degree, was diffuse, and many of these inflammatory cells were distorted or disintegrating. The tissues adjacent the gland were edematous and infiltrated with fibrin and neutrophilic leucocytes and lymphocytes (fig. 12). The fibroblasts were large, and had hyperchromatic nuclei and prominent nucleoli, changes that indicated active growth. The small blood vessels in and about the thyroid gland were filled with thrombi composed of red blood cells. No organization had occurred at this interval.

Portions of the parathyroids showed distorted epithelial cells, and were infiltrated with fibrin and a few neutrophilic leucocytes.

The striated muscle adjacent the gland still manifested an interfiber edema and contained scattered leucocytes. The tracheal epithelium showed no histological changes.

The Gland at Two Weeks.—The condition of the thyroid gland at this time resembled that observed at one week. A few disintegrating follicles composed of degenerated cells were still visible in the periphery of the gland. Throughout the gland there were also some groups of apparently viable thyroid cells no longer in follicular arrangement. These cells had an abundant granular cytoplasm.

Fibrin was still abundant in the interstitial spaces and within the remnants of the follicles. Granulation tissue was not evident. The mild inflammatory reaction was now of the chronic type and was associated with early fibroblastic proliferation particularly marked at the periphery of the lobes. A few of the macrophages contained blood pigment.

Alterations in the arteries were now quite advanced. Their walls were infiltrated with fibrin and neutrophilic leucocytes and the endothelium was usually desquamated. Many of these vessels were throm-

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FIG. 5. Thyroid gland from rat sacrificed 48 hours after the injection of 875 microcuries of I^{131}. Follicular disruption, acute inflammation and destruction of epithelial cells characterized the gland at this time. Hematoxylin and eosin; ×80.

FIG. 6. Higher magnification of gland shown in Fig. 4. Note the pyknotic cells, lack of follicular organization, and deposition of fibrin. Hematoxylin and eosin; ×400.

FIG. 7. Another region of gland shown in Fig. 4. Swollen, vacuolated, epithelial cells from the upper pole of the gland in an early stage of the degenerative process. Hematoxylin and eosin; ×400.

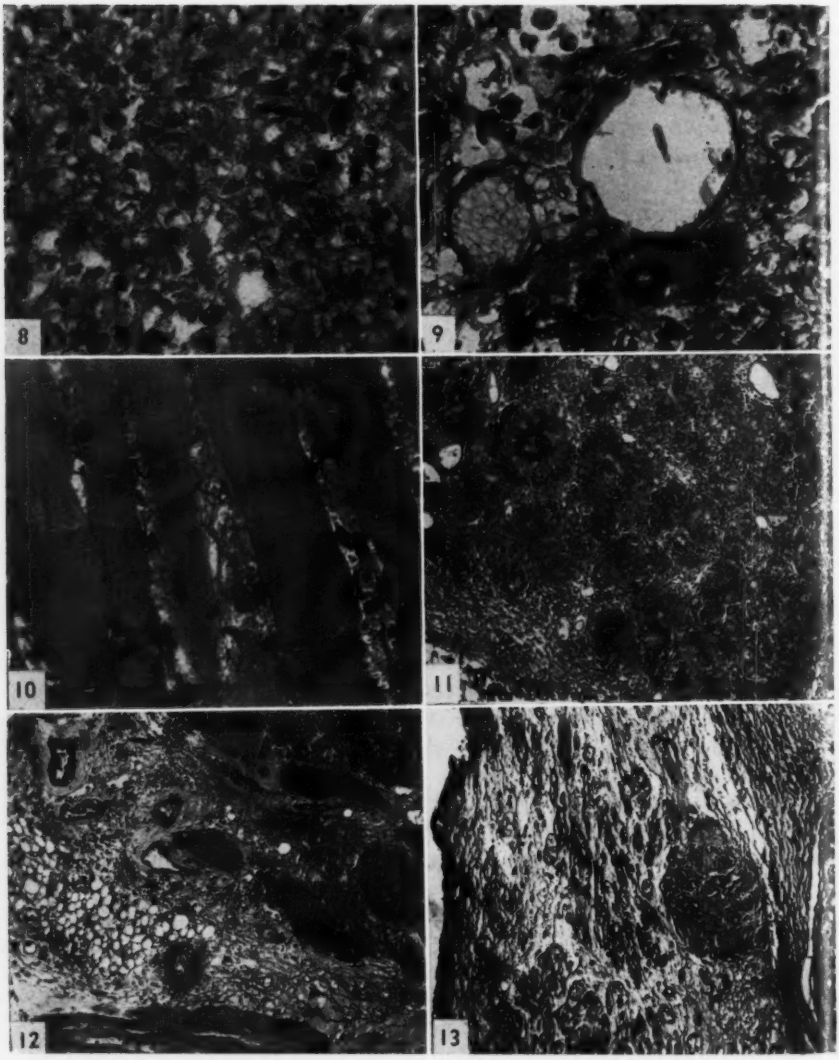

FIG. 8. Thyroid gland from rat sacrificed 72 hours after the injection of 875 microcuries of I^{131}. At this time, only the stromal framework identified the remains of the follicles. Degenerating epithelial cells, fragmented nuclei, macrophages, and leucocytes may be seen in the heavily congested gland. Hematoxylin and eosin; ×400.

FIG. 9. Follicular "ghosts" from gland shown in Fig. 7. Hematoxylin and eosin; ×400.

FIG. 10. Striated muscle adjacent thyroid gland of rat sacrificed 72 hours after the injection of 875 microcuries of I^{131}. Swelling and loss of striation in portion of the individual muscle fibers, interfiber edema, and inflammation can be observed. Hematoxylin and eosin; ×760.

FIG. 11. Thyroid gland of rat sacrificed one week after the injection of 875 micro-

bosed, and organization had already occurred in some of the thrombi. Similar vascular changes as well as a leucocytic infiltration, were found in the adjacent striated muscles.

Changes at Three Weeks (Fig. 13.).—At this time, the thyroid was largely a fibrinous mass containing a few scattered, degenerated thyroid cells. Fibrous organization had occurred, with the formation of numerous small, capillary channels. The mild, chronic, diffuse, inflammatory reaction persisted, and many pigment-laden macrophages were present.

The periglandular tissue was less edematous than at the two-week interval, but was more heavily infiltrated with lymphocytes, macrophages, and neutrophilic leucocytes. The surrounding fat cells had enlarged, hyperchromatic nuclei. The vascular damage in as well as adjacent the gland closely resembled that described for the two-week study.

Portions of several parathyroid glands were replaced by new collagenous tissue and were invaded by leucocytes. The capsules of these glands were also thickened and edematous. Many of the parathyroid glands, however, appeared quite normal.

The tracheal epithelium had lost its ciliated border in the region adjacent the thyroid and, in addition, thinning of the epithelium, with nuclear pyknosis, was observed.

The adjacent, striated muscle fibers were normal in appearance.

Condition of the Gland at One Month (Fig. 14).—The histological appearance of the gland at this time was quite similar to that found at three weeks. A diffuse, fine, collagenous stroma now permeated the gland, and small, dense whorls of new collagenous tissue were present.

Condition of the Gland at Six and Eight Months (Figs. 15–18).—The region formerly occupied by the thyroid gland now consisted of a narrow band of hyalinized, collagenous, fibrous tissue in which were embedded the recurrent laryngeal nerve and the parathyroid glands (fig. 15). Twelve rats were examined at six and eight months after the injection of the I^{131} and, in all but one, this region did not contain a single identifiable thyroid epithelial cell.

In the single animal mentioned above, a few scattered, epithelial-like cells were found within the hyalinized area. These round, or polyhedral, cells had a granular, faintly eosinophilic, cytoplasm which was imperfectly outlined. Their round, vesicular nuclei were not un-

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curies of I^{131}. Note the condition of the thyroid parenchyma, the congestion, inflammation, and heavy fibrin deposition. Hematoxylin and eosin; ×80.

FIG. 12. Upper pole of gland shown in Fig. 10. The periglandular inflammation and perivascular reaction may be seen. Hematoxylin and eosin; ×80.

FIG. 13. Thyroid gland of rat sacrificed three weeks after the injection of 875 microcuries of I^{131}. Fibroblastic proliferation and deposition of fibrous tissue are observed at this time. Only an occasional intact epithelial nucleus can be found at this time. Hematoxylin and eosin; ×80.

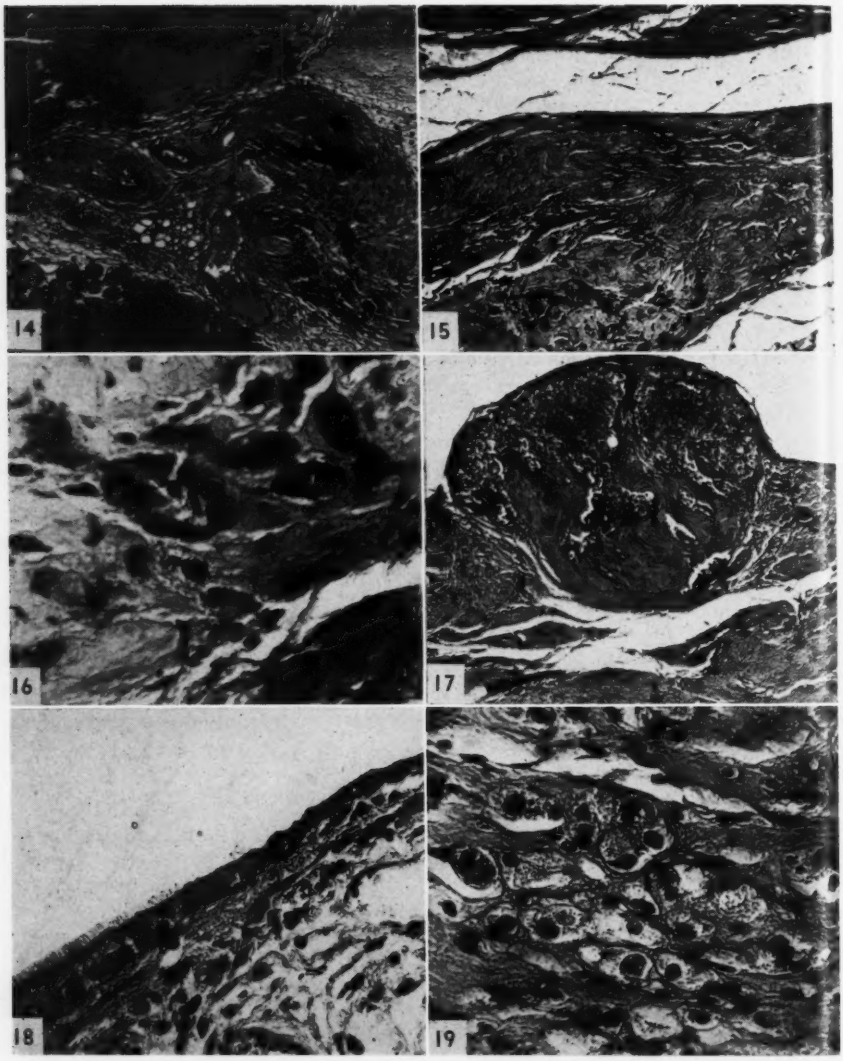

FIG. 14. Thyroid gland from rat sacrificed four weeks after the injection of 875 microcuries of I^{131}. Note the newly formed, dense, fibrous tissue in the lower right hand corner of the picture, and fibrous proliferation in the vascular walls. The inflammation and edema in the adjacent muscle (upper and lower left) has subsided. Hematoxylin and eosin; ×80.

FIG. 15. The thyroid gland from rat sacrificed six months after the injection of 875 microcuries of I^{131}. Note the dark pigment deposits in the hyalinized, fibrous tissue which has completely replaced all epithelial elements of the thyroid gland. Hematoxylin and eosin; ×80.

FIG. 16. Pigment-laden macrophages seen in Fig. 14. Hematoxylin and eosin; ×400.

FIG. 17. Parathyroid gland from animal sacrificed eight months after the injection

usual. At the periphery of the fibrous band, there was an occasional small thyroid follicle containing a small amount of colloid-like material. These acini were lined by squamous cells (generally 3–4) with compact, dense nuclei.

Numerous deposits of dark brown-staining pigment were a prominent feature. These were scattered throughout the dense connective tissue which had replaced the thyroid gland (figs. 15–16).

The parathyroid glands appeared normal except for fibrous thickening of the capsule and widening of the fibrous trabeculae (fig. 17).

The tracheal epithelium in the region adjacent the destroyed thyroid gland had not regained its normal appearance. The epithelial border consisted of simple, nonciliated, cuboidal cells with small, pyknotic nuclei. Loss of glandular elements and fibrous replacement were observed in the mucosal and submucosal layers (figs. 18).

II. *Rats that Received a Single Injection of 525 Microcuries of I^{131}*

The thyroid glands of rats were examined at 12 hours, one day, two days, three days, one week, one month, five months, six months, and eight months after the injection of 525 microcuries of I^{131} (Table 1). The changes observed in the *early intervals* were indistinguishable from those of rats sacrificed at corresponding intervals after the injection of 875 microcuries. Consequently, a description of the changes observed at five to eight months only is given below.

Condition of the Gland at 5–8 Months (Figs. 19–22).—The thyroid gland was thin, elongated, and consisted largely of amorphous, hyaline-fibrous tissue in which were embedded numerous clumps of dark pigment, contained largely in degenerated macrophages. Within the area formerly occupied by the gland were scattered thyroid epithelial cells with distorted nuclei (fig. 19). At the two poles of this area, thyroid cells were more numerous, and some of them had formed small follicles which were lined by a few squamous, atrophic cells. A few of these follicles contained granular, vacuolated colloid. The scattered thyroid cells were quite large and contained variable amounts of granular eosinophilic cytoplasm. Their nuclei exhibited extreme pleomorphism and hyperchromasy (fig. 19). Many of these nuclei contained extremely large nucleoli. Mitoses were absent in these thyroid cells, which resembled the so-called Hürthle cells of human thyroid glands (10).

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of 875 microcuries of I^{131}. The epithelial cells appear normal, but a considerable portion of the gland has been replaced with fibrous tissue. Hematoxylin and eosin; ×80.

FIG. 18. Nuclear pyknosis, reduction of epithelial height, and loss of cilia from tracheal epithelium adjacent the destroyed thyroid gland. Six months after injection of 875 microcuries of I^{131}. Hematoxylin and eosin; ×400.

FIG. 19. Surviving epithelial cells in thyroid of rat sacrificed six months after the injection of 525 microcuries of I^{131}. Note the large, granular cells with hyperchromatic and pleomorphic nuclei and large nucleoli. Hematoxylin and eosin; ×400.

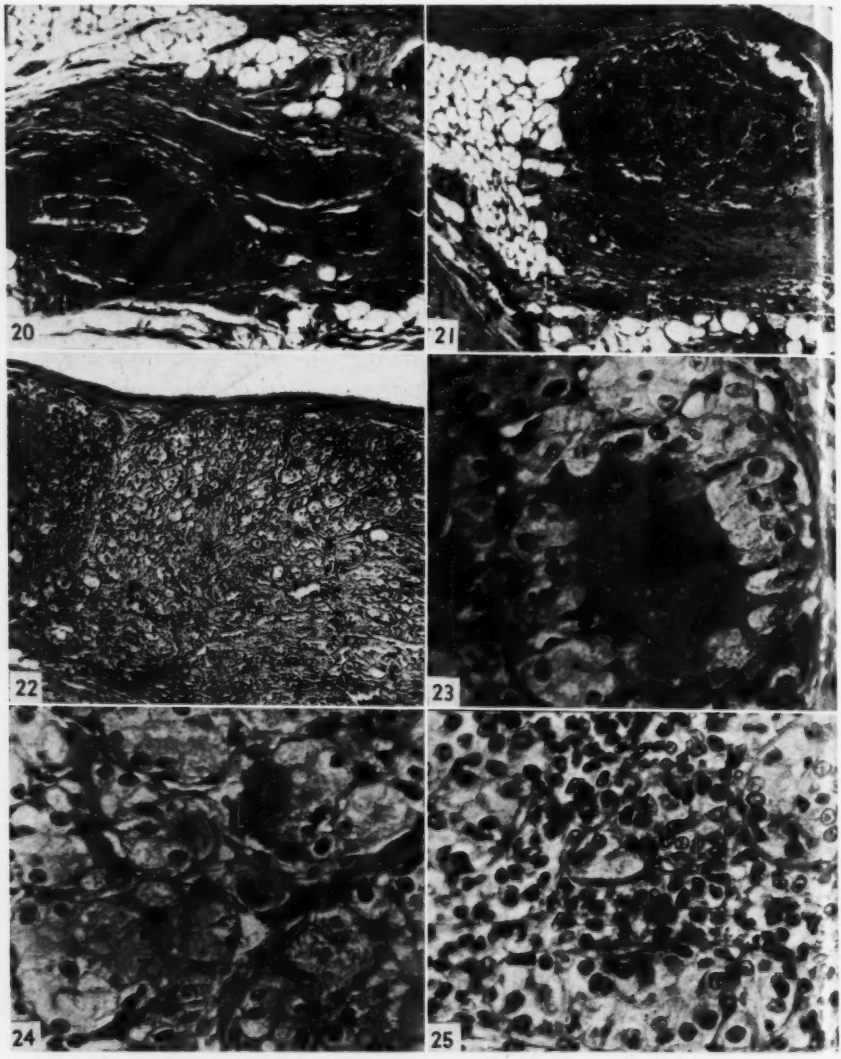

FIG. 20. Thickened, hyalinized, fibrous wall of artery in thyroid of rat sacrificed six months after the injection of 525 microcuries of I^{131}. A portion of the recurrent laryngeal nerve is seen just to the right of center. Hematoxylin and eosin; ×80.

FIG. 21. Parathyroid from rat injected with 525 microcuries of I^{131} six months previously. The epithelial cells appear normal. Below the parathyroid is a small cluster of thyroid cells similar to those shown in Fig. 18. Hematoxylin and eosin; ×80.

FIG. 22. Thyroid gland from rat sacrificed eight months after injection of 525 microcuries of I^{131}. Many cells similar to those seen in Fig. 18 are seen at this time, apparently proliferating in a peripheral direction. A portion of the intact parathyroid is seen in the upper left. Hematoxylin and eosin; ×80.

FIG. 23. Vacuolation, swelling, and desquamation of epithelial cells in the thyroid

In two animals, the thyroid resembled the hyperplastic condition seen after long-term treatment of normal animals with propylthiouracil, i.e., a microfollicular macrocellular structure. Scanty, pale colloid was present.

A consistent finding in the 18 rats of this group was the thickened, hyaline-fibrous walls of the arteries adjacent and within the glands (fig. 20).

In several glands, the fibrous tissue which had replaced the thyroid parenchyma had assumed a whorl-like form.

In some rats, the cartilagenous rings adjacent the altered thyroid were degenerated, as evidenced by loss of cartilage cells and fibrillary degeneration of the chondromucin.

The parathyroids were not unusual (fig. 21) but the trachea revealed alterations. The tracheal epithelium adjacent the thyroid was reduced to a layer of low, cuboidal, nonciliated cells with pyknotic nuclei. The submucosal and mucosal layers showed fibrosis and loss of glandular elements.

In the rats examined at *eight* months, the thyroid cells appeared more numerous than in those examined at five or six months, particularly at the periphery and poles of the gland (fig. 22). Occasional small follicles were found, some of which contained a small amount of colloid. No calcification was noted in the thyroid area or in the adjacent cartilage.

III. *Rats Injected with 300 Microcuries*

Six and Twelve Hours.—No histological evidence of damage was found in any of the tissues of rats that had been sacrificed at these intervals.

Twenty-four Hours (Figs. 23–24).—Visible changes in the thyroid gland were first found at this time. The follicular cells were markedly swollen (fig. 23), and some were even several times their normal size. This resulted, in many cases, in an almost complete obliteration of the follicular lumen (fig. 24). Vacuolation was prominent in the cytoplasm of these swollen cells (fig. 24). The normal granular appearance of the cytoplasm was often lost. In many cells, there was wrinkling or folding of the nuclear membrane and clumping of chromatin material, leading to nuclear pyknosis. By the time 24 hours had elapsed, epithelial desquamation into follicular lumens was quite ex-

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gland of rat sacrificed 24 hours after the injection of 300 microcuries of I^{131}. Hematoxylin and eosin; ×400.

FIG. 24. Obliteration of follicular lumens by the swollen epithelial cells of the thyroid gland of rat 24 hours after the injection of 300 microcuries of I^{131}. Note the swollen endothelial cells of the capillary a little to the right of center. Hematoxylin and eosin; ×400.

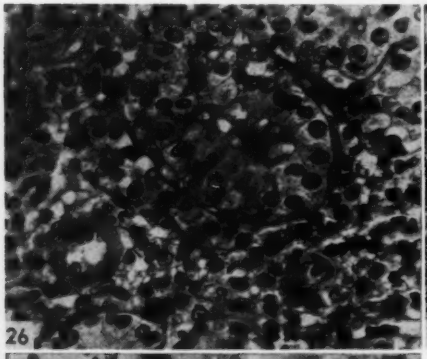
FIG. 25. Surviving swollen epithelial cells from the thyroid of rat sacrificed 72 hours after the injection of 300 microcuries of I^{131}. Hematoxylin and eosin; ×400.

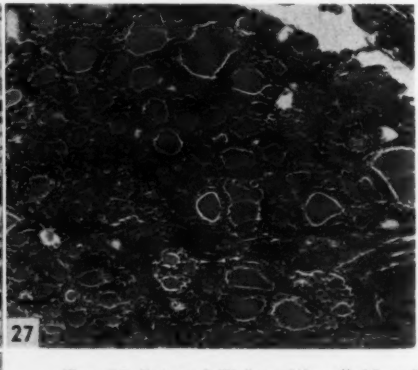
tensive. The arteries and capillaries were also affected; their endothelial cells were found bulging into their lumens, and the media were swollen (fig. 23). No evidence of leucocytic infiltration or inflammatory reaction in the thyroid gland was observed.

Forty-eight Hours.—The extensive edema present at this time was accompanied by a cellular infiltration of lymphocytes, neutrophilic leucocytes, and macrophages. Vascular changes of the type described above (24 hours) were now more severe in degree. In the thyroid gland of one rat (eight in all were examined at this interval), an extensive perivascular deposition of fibrin was found.

A few pyknotic nuclei were observed in the parathyroid of one animal, but otherwise this gland appeared undamaged.

Seventy-two Hours (*Figs. 25–26*).—The thyroid gland now presented a picture of more advanced degeneration. Many follicles were in various stages of degeneration, and colloid appeared to be flowing out between the damaged cells. The acinar cells in the damaged follicles were pyknotic and fragmented. Subcapsular and interstitial edema were more extensive at this time than at 48 hours. The glands





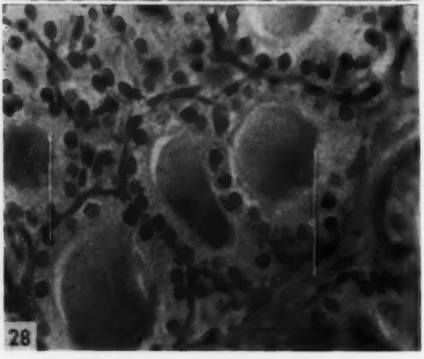


FIG. 26. Intact follicles with colloid and high columnar epithelial cells from gland shown in Fig. 24. Note distorted arteriole at lower left. Hematoxylin and eosin; ×400.

FIG. 27. Thyroid gland from rat sacrificed eight months after the injection of 300 microcuries of I^{131}. Normal follicular structure is seen at this time. The lining cells are cuboidal with condensed, small nuclei. Hematoxylin and eosin; ×80.

FIG. 28. Thyroid gland from rat sacrificed one week after the injection of 18 microcuries of I^{131}. The epithelium, which varies from cuboidal to columnar, is similar to that of the normal adult rat maintained on the diet used here. Hematoxylin and eosin; ×400.

were infiltrated with fibrin, lymphocytes, neutrophilic leucocytes, and macrophages (fig. 25). There was abundant cellular debris. Scattered throughout were some follicles which presented no histological evidence of severe epithelial damage and which were filled with deep-staining, vacuolated, eosinophilic colloid (fig. 26). Many of these surviving follicles seemed to be in an active secretory phase as judged by cell height and the presence of intracellular colloid droplets.

Eight Days.—At this time, the edema of the gland was greatly reduced and very little of the interstitial cellular reaction was evident. Most of the debris had disappeared, and many intact follicles were surrounded by newly formed fibrous tissue. These follicles contained colloid, and were lined by typical cuboidal, or low columnar, epithelial cells. The arteries and capillaries had regained their normal appearance although thrombosed vessels were still present. No damage to the recurrent laryngeal nerve was observed.

Four Months.—The glands were small and lobular in appearance as a result of the presence of irregular, fibrous septa. Many typical follicles were contained in these lobules. Many thyroid epithelial cells were also arranged in irregular groups which, in some cases, formed distorted follicles. A few of these distorted follicles contained colloid. The thyroid cells lining these follicles, and their nuclei, were variable in size and shape. A few nuclei were very large and quite hyperchromatic. The cytoplasm was granular, varied in amount, and was poorly outlined in most instances.

The parathyroid glands appeared normal, but were surrounded by a thickened, connective-tissue capsule.

The connective tissue adjacent the tracheal rings and muscle showed evidence of fibrosis and was infiltrated with mast cells.

Reactions Observed at Eight Months (*Fig. 27*).—At this late period the gland, although not pathological, differed somewhat from the typical rat thyroid. The follicles were normal in size and shape, and contained dense, eosinophilic colloid. The cells lining the follicles were quite uniform, or low-columnar or cuboidal in height. Their nuclei were more compact than normal, and stained more heavily with hematoxylin. No alteration was observed in the blood vessels. The interfollicular spaces contained increased amounts of fibrous tissue in which were found macrophages and mast cells.

The parathyroid glands were not abnormal.

IV. *Rats that Received a Single Injection of 18 Microcuries of Carrier-Free* I^{131}

No evidence of damage to the thyroid or contiguous tissues was found in rats examined at six, 12, 24, 72, 216 hours, and one month after the injection of 18 microcuries of I^{131} (fig. 28).

V. *The Effect of Injection of Various Doses of I^{131} on Liver, Kidney, and Other Tissues*

No pathological changes were detected in the liver at any time after the injection of the four doses of I^{131} used in this study.[5] The kidneys of a few of the animals revealed slight glomerular congestion and slight cloudy swelling up to one week after single injections of 525 and 875 microcuries. The kidneys were found normal at intervals after one week. No pathological alterations were observed in other tissues.

DISCUSSION

The present study is based on the examination of the thyroid glands of 250 rats which were sacrificed at intervals from six hours to eight months after single injections of four different doses of radioiodine. The changes observed in the gland may be considered in several stages: (1) Degeneration and necrosis of epithelial cells with follicular disruption; (2) vascular degeneration and thrombosis; (3) acute and chronic inflammation; (4) fibrous organization; and (5) epithelial regeneration.

The earliest observable cytological changes consisted of cytoplasmic vacuolation and swelling. Nuclear changes leading to pyknosis and karyorrhexis also occurred quite early. These degenerative effects of radiation are similar to those reported by many investigators in other tissues damaged by radiation (Warren, 1944; Bloom, 1948; and Hertwig, 1913).

Injury to the thyroid epithelium is followed by an inflammatory response consisting of edema and infiltration with fibrin and leucocytes. At this time, evidence of follicular disruption was seen. The reparative process was initiated quite early (approximately one week after the injection of damaging dosages) with the appearance of proliferating fibroblasts. It is apparent that the injurious effect of highly localized radiation results in an inflammatory reaction that is not unique, but one that closely resembles the response to other types of noxious stimuli.

At the early intervals, the major differences in the reactions observed with the various doses were of degree and not of kind. Although the minimum amount of radiation necessary to induce histological changes in the thyroid gland was not determined, it is clear that the delivery of an estimated dose of 12,000 reps to a point at the center of the gland over a period of nine days (rats injected with 18 microcuries) is insufficient (Table 1). This dose is somewhat less than one quarter the amount delivered to the thyroid gland during the 24 hours after the injection of 300 microcuries—a dose that visibly damaged the gland (53,000 reps, Table 1).

[5] An increase in the amount of histologically demonstrable fat has been noted in the livers of a few rats 11 months after injection of 875 microcuries I^{131}. This is being more extensively studied.

The degree of initial destruction appears to determine the final picture in the gland. Thus 6–8 months after the injection of 875 microcuries, only a thin, hyalinized, collagenous band, devoid of all thyroid epithelial elements, was found in the thyroid region. In contrast to this total destruction of the thyroid gland were the results obtained with 300 and 525 microcuries. In the early intervals after injection of 300 microcuries, a large number of epithelial cells were apparently undamaged. Five–eight months later, normal glandular structure was established and normal physiological function was regained (Goldberg and Chaikoff, following paper).

Although the histopathological changes found in the early intervals, in rats injected with either 525 or 875 microcuries, were indistinguishable, it was surprising to note that marked differences existed in these animals at the end of 6–8 months. In those that received 875 microcuries, no demonstrable thyroid cells were present at 6–8 months. But in those that received 525 microcuries, numerous viable thyroid cells were found in 6–8 months. These cells had large, irregular nuclei, which exhibited hyperchromasy and pleomorphism and, in addition, showed cytoplasmic alterations. The cytoplasm was eosinophilic and granular, and often was not clearly delineated by a cell membrane. These cells resemble somewhat the so-called Hürthle cells described in certain human thyroid glands (Willis, 1948). Although mitoses were not observed, it seems evident that these cells were proliferating since they were more numerous at eight months than at five months and since their nuclei resembled those of active cells. These residual thyroid cells were found to be proliferating largely at the poles of the glands. This can probably be explained by the fact that the polar regions received less radiation than did the center of the glands. It also seemed evident that the dense, hyalinized, collagenous tissue in the center of the glands was a factor preventing central regeneration. The cells proliferated towards the periphery in the direction of least resistance. Although there was a slight tendency of the cells to form small, irregular follicles, they were not functioning in their normal capacity, i.e., not producing thyroxine (Goldberg and Chaikoff, following paper). The question as to whether or not these degenerated cells have the ability to concentrate and bind iodine organically is now being investigated.

The significance of the eosinophilic cytoplasmic change (Hürthle cells) seen in human thyroid glands, in a variety of pathologic conditions, is not understood. Because radiation is known to produce minute nuclear changes, and because these may be associated with untoward genetic mutations, this late cellular proliferation is being further investigated in animals receiving doses of I^{131} too small to cause complete thyroid destruction, but large enough to prevent normal regeneration.

In a recent communication (Feller *et al.*, 1949) criteria based on

the specialized function of the thyroid gland with respect to iodine metabolism have been employed to estimate toxicity of injected I^{131}. These were: (1) level of plasma protein-bound iodine; (2) uptake and retention of the initially injected I^{131}; (3) thyroid iodine, organic as well as inorganic; and (4) uptake of a second dose of labeled iodine.

The earliest *metabolic* indicator of decreased glandular function was a drop in the level of plasma protein-bound iodine. This was observed as early as 24 hours after the injection of 875 microcuries of I^{131} (150,000 reps delivered to gland (Feller *et al.*, 1949)). It is shown here, however, that *histological* evidence of damage to the thyroid appears even earlier. As early as 12 hours after an injection of 875 microcuries of I^{131}, consistent cytological changes were observed in the thyroid epithelium.

The rate of loss of the injected I^{131} by the gland was not the same for 18, 300, and 875 microcuries of I^{131} (fig. 1), but roughly paralleled the degrees of degeneration and inflammation observed at 48 and 72 hours. In the case of the 875-microcurie injection, only the follicular outlines and scattered, single, degenerating thyroid cells remained at the end of 72 hours; at this interval, less than *one per cent* of the injected dose remained in the gland. On the other hand, the retention of 23 per cent of the injected I^{131} in the gland at 72 hours after the injection of 300 microcuries corresponded to the histological finding of many intact follicles. Animals injected with 18 microcuries of I^{131} retained 37 per cent of the administered dose at 72 hours (fig. 1).

It is apparent that radioiodine can be used to effect total thyroidectomy in the rat without major deleterious side effects. This has several obvious advantages over the surgical method. These are: (1) simplicity; (2) destruction of any aberrant thyroidal tissue that might be present; and (3) preservation of the parathyroids without recourse to minute dissection and transplantation. An injection of 875 microcuries has been found adequate for complete destruction of the thyroid gland in the rat of the Long-Evans strain maintained under the dietary conditions described here.

The resistance of the parathyroid glands to I^{131} injection is quite impressive. Relatively little damage was observed in these glands even in rats injected with 875 microcuries of I^{131}. Their survival might be expected because of the comparatively short paths of the beta rays in tissues and the differential in iodine concentrating capacity of the thyroid and parathyroid cells.The survival of parathyroids has previously been demonstrated in dogs (Hamilton, 1942) injected with large doses of I^{131}, and in the new-born rat (Goldberg and Chaikoff, 1949) in which complete destruction of the thyroid gland by means of I^{131} was established. Gorbman (1947), on the other hand, has reported destruction of the parathyroids in mice which received sufficient radiation to obliterate the thyroid.

The occurrence of degenerative changes (tumorous) in the trachea of mice injected with large doses of I^{131} has been reported by Gorbman (1949). Fibrous changes and loss of glandular epithelial elements were observed in the rats examined in this study.

SUMMARY

Rats of the Long-Evans strain, weighing about 250 gm., were given single, intraperitoneal injections of 18, 300, 525, or 875 microcuries I^{131}. Autopsies were performed on groups at short intervals up to three days, then weekly up to one month, and thereafter at five, six, and eight months after the injection.

No evidence of damage to any tissue was observed at any of the intervals after the injection of 18 microcuries I^{131}.

A progressive degenerative process was observed in the thyroid glands of animals that received 300 or 525 microcuries I^{131}. This was arbitrarily divided into five stages: (1) degeneration and necrosis of epithelial cells with follicular disruption; (2) vascular degeneration and thrombosis; (3) acute and chronic inflammatory changes; (4) fibrous organization; and (5) epithelial regeneration.

The thyroids of rats that had been injected with 875 microcuries responded in a similar fashion except that no epithelial regeneration occurred. No thyroid epithelial elements were found in rats eight months after a single injection of 875 microcuries of I^{131}.

Cytological changes were detected in thyroid glands as early as 12 hours after single injections of 525 or 875 microcuries of I^{131}, and as early as 24 hours after 300 microcuries.

Regeneration to normal thyroid structure was found in animals which had been injected with 300 microcuries of I^{131}.

Five to eight months after the injection of 525 microcuries of I^{131} *atypical* thyroid cells, single and in groups, were observed. These cells showed some resemblance to the so-called Hürthle cells found in certain thyroid lesions in man.

Parathyroid glands were found in all animals that had been injected with I^{131}, and these glands appeared viable. Some fibrosis had occurred in the peripheral portions of the glands, particularly when 525 and 875 microcuries of I^{131} were injected.

Evidence of tracheal damage, and slight reversible changes in the kidneys, were noted. No pathological effects were observed in the livers of these animals.

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THE CYTOLOGICAL CHANGES THAT OCCUR IN THE ANTERIOR PITUITARY GLANDS OF RATS INJECTED WITH VARIOUS DOSES OF I^{131} AND THEIR SIGNIFICANCE IN THE ESTIMATION OF THYROID FUNCTION[1]

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THE VALUE of a cytological study of the anterior pituitary as a tool for the estimation of thyroid activity has been pointed out by Griesbach *et al.* (1941, 1943, 1945, 1946, 1949). The percentage of basophilic elements, which are considered the site of thyroid stimulating hormone production, increases in the presence of a slight thyroxine deficiency. For example, there is a measurable increase in the percentage of pituitary basophils in goitrogen-treated rats when the amount of injected l-thyroxine equivalent to their daily requirement is decreased by as little as 0.1 microgram per 100 gm. of body weight (Griesbach *et al.*, 1949). A more severe thyroxine deficiency results in an increase up to eight-fold in the percentage of these cells and their transition into a signet-ring type of cell. The acidophilic elements, on the other hand, become sparsely granulated and decrease in percentage only when the thyroid deficiency is quite severe. A distinguishing feature of complete thyroid deficiency is the almost complete absence of *granulated* acidophils (referred to as complete degranulation). Thus, an acidophil count can be used to differentiate between (1) a complete lack of thyroid hormone, and (2) the presence of very slight amounts of this hormone.

The purpose of the present study was to provide information on the functional activity of thyroid glands that had been internally irradiated with I^{131}. For the aforementioned reasons, anterior pituitary cytology was employed in the estimation of residual thyroid function.

EXPERIMENTAL

The pituitaries studied here were removed from normal male rats and male rats injected with I^{131}. The various groups are described in the preceding report (Goldberg *et al.*, preceding paper). The rats were exsanguinated and their pituitaries rapidly removed thereafter.

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[2] Atomic Energy Commission Fellow.

TABLE 1. DISTRIBUTION OF ACIDOPHILS, BASOPHILS AND CHROMOPHOBES IN THE ANTERIOR PITUITARY OF RATS INJECTED WITH VARIOUS DOSES OF I^{131}

I^{131} injected	Interval after injection sacrificed	Anterior pituitary‡		
		Acidophils	Basophils	Chromophobes
none (controls)*	—	per cent of total 43.2	per cent of total 5.1	per cent of total 51.7
	hours			
875	6	42.1	4.3	53.6
875	12	44.7	5.7	49.6
875	24	43.8	6.1	50.1
875	24	44.6	6.4	49.0
875	72	41.1	5.4	53.5
875	72	45.7	5.9	48.4
	weeks			
875	1	32.8	12.9	54.3
875	2	16.3	18.4	65.3
875	2	12.2	26.6	61.2
875	3	0.9	27.9	71.2
875	3	0.4	31.3	68.3
875	4	0.7	29.4	69.9
875	4	1.1	21.0	77.9
	months			
875	6	1.3	16.4	82.3
875	6	0.7	19.2	80.1
875	6	0.8	18.4	81.8
875	8	0.8	19.7	79.5
875	8	1.2	18.4	80.4
875	8	1.1	20.1	78.8
875	8	1.0	19.3	80.7
525	6	1.2	16.3	82.5
525	6	1.0	13.2	85.8
525	6	1.4	14.7	83.9
525	6	1.2	10.1	88.7
525	8	0.9	14.3	84.8
525	8	1.4	14.1	84.5
525	8	1.2	17.3	81.5
525	8	1.1	22.7	76.2
525†	8	45.7	8.3	45.0
525†	8	37.1	14.1	48.8
	weeks			
300	1	38.4	9.4	52.2
300	1	36.7	8.3	54.0
300	2	24.3	11.1	64.6
300	2	29.7	12.3	57.0
300	3	30.2	13.4	56.4
300	3	25.4	14.3	60.3
300	4	46.1	17.4	36.5
300	4	43.2	16.9	39.9
	months			
300	5	41.3	5.4	53.3
300	5	40.8	7.3	51.9
300	5	42.4	6.9	50.7
300	8	32.3	4.7	63.0
300	8	44.3	5.1	50.6
300	8	45.1	4.7	50.2
300	8	42.7	5.3	52.0
	hours			
18	12	41.4	5.3	53.3
18	12	43.7	6.2	50.1
18	72	42.9	5.8	51.3
18	72	41.9	4.3	53.8
	month			
18	1	45.3	4.7	50.0
18	1	42.4	5.9	51.7
18	1	44.6	6.4	50.0
18	1	44.8	4.9	50.3

* Values are averages of 43,000 counts made on 20 animals.
† See text.
‡ At least 2000 cells were counted per animal.

These glands were immediately fixed for 24 hours in a solution containing nine parts $HgCl_2$ (saturated) and one part 40 per cent formaldehyde. They were dehydrated in alcohol, cleared in cedar wood oil, embedded in paraffin, and sectioned serially at 2–3 micra. The sections were stained by Martins' hematoxylin-Mallory stain (Martins, 1933). Differential cell counts were made by a method similar to those described by Purves and Griesbach (1946) and Floderus (1944). At least 2000 cells per gland were counted.

A. CYTOLOGY OF THE ANTERIOR PITUITARIES OF NORMAL AND I^{131}-INJECTED RATS

The pituitaries of all rats (250 Long-Evans males) were examined histologically; cell counts were made on 73 rats randomly selected from the various groups (Table 1).

1. The Normal Rat (Fig. 1 and Table 1)

The normal histology of the rat anterior pituitary has been described by several investigators. Three principal types of cells are present: acidophils, basophils, and chromophobes. Romeis (1940) described six cell types. In several species, including the rat, he described two types of basophils (beta and delta), two types of acidophils (alpha and epsilon), chromophobes (gamma), and an undifferentiated cell. We investigated his resorcin-fuchsin staining technique for demonstrating these cell types, but found that the duration of staining with resorcin-fuchsin influenced the number of beta cells

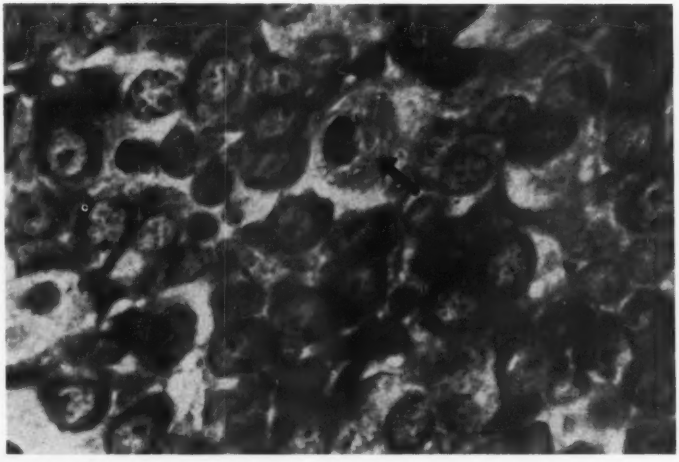

FIG. 1. Anterior pituitary gland from normal male rat. Note the numerous acidophils (dark cytoplasm). Arrow points to large basophil with pyknotic nucleus and small golgi apparatus (seen as negative image). Martins; ×1200.

(Romeis classification). Although we did observe two tinctorial types of acidophils (Romeis' alpha and epsilon cell-types), they did not respond differently to thyroid destruction by I^{131}. For these reasons, Romeis' technique was not used in the present investigation.

The anterior pituitary glands of normal rats of the Long-Evans strain (fig. 1) contain approximately 4–7 per cent basophils (Table 1). These round, or polyhedral, cells have a blue staining granular cytoplasm and frequently contain pyknotic nuclei with a single large nucleolus. A negative image of the golgi apparatus is seen as a nonstaining ring surrounding a dark staining, granular area between the nucleus and the cell membrane. Infrequently, a basophil containing a clear hyaline vacuole is seen, but these constitute, in the normal adult rat, only a small per cent of the total number of basophils.

Acidophils constitute about 41 to 46 per cent of the total cells in the normal rat anterior pituitary (fig. 1 and Table 1). These cells are smaller than the basophils. Their coarse, refractile granules can be distinctly seen only in sections one or two microns thick. The negative image of the golgi apparatus appears as a light, clear cap to one side of the nucleus. The latter contains one or two large nucleoli.

The chromophobes (48–53 per cent of the total cell count) are about the same size as the acidophils. The chromophobes are irregular in outline, have poorly defined cell boundaries, and contain a light, homogeneous, nonstaining, finely granular cytoplasm. Often only the nuclei can be perceived.

2. Rats Injected with 18 Microcuries[3] of I^{131} (Table 1)

No alterations either in cell morphology or in percentage distribution were noted in the anterior pituitaries of rats examined from 12 hours to one month after the injection of 18 microcuries of I^{131}.

3. Rats Injected with 300, 525, and 875 Microcuries of I^{131}

At End of One Week.—Demonstrable changes were first noted in the pituitary one week after the injection of 300, 525, or 875 microcuries of I^{131}. At this time, a definite decrease in the amount of granulation in the acidophils, as well as in their percentage, was observed (Table 1). But the most striking change occurred in the basophils which, already measurably increased in number, had undergone marked hypertrophy and contained enlarged golgi apparatuses and vesicular nuclei. Mitotic figures (rare in the normal pituitary) were observed in the basophils, indicating that an actual increase in their number was occurring.

At End of Two Weeks.—Two weeks after the injection of 300, 525, or 875 microcuries I^{131}, a more pronounced decrease (Table 1) in the number of acidophils was observed, those remaining being poorly granulated. The basophils were greatly increased in size and number,

[3] For value of the microcurie, see footnote 4 of preceding paper.

and many of them contained pale blue, hyaline vacuoles. The vacuoles, for the most part, were large and single. However, a few of the cells showed several very small vacuoles. Also present were many deeply staining, coarsely granulated basophils with no vacuolation.

At End of Three Weeks.—The acidophils had almost completely disappeared in animals that had received 525 or 875 microcuries of I^{131}, whereas in those that received 300 microcuries of I^{131}, they comprised approximately 25–30 per cent of the total cells. The appearance of the glands containing practically no acidophils was characteristic even under macroscopic inspection of the slides. Their bluish cast

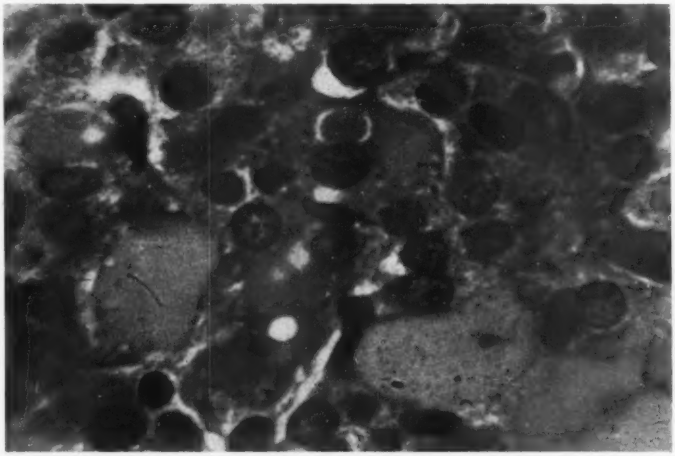

FIG. 2. Anterior pituitary gland from rat sacrificed one month after the injection of 875 microcuries I^{131}. Note the vacuolated basophils with large golgi apparatuses and vesicular round nuclei. Acidophils are almost completely degranulated (compare with fig. 1). Martins; ×1200.

readily distinguished them from those containing granulated acidophils.

The basophils were numerous and extremely large, revealing extensive hyaline vacuolation. These cells usually contained one large vacuole, but two or more smaller vacuoles in a cell were not uncommon.

The chromophobic cells exhibited extremely large acidophilic nucleoli, some with diameters roughly a third as great as those of the nuclei.

At End of Four Weeks (*Fig. 2*).—The findings at this time were similar to those observed at three weeks except that the hyaline vacuoles of the basophils were larger in size and were usually single. In these basophils, the nuclei and the golgi apparatuses were, as a rule, compressed into thin rims of cytoplasm at the periphery of the cells. These are the signet-ring basophil cells.

Many non-vacuolated basophils filled with coarse, deep blue granules were also present. The golgi apparatus of these cells was not enlarged and the nuclei were often pyknotic in appearance.

At End of 5–8 Months.—At this time, the pituitaries of animals that had received injections of 300 microcuries of I^{131} revealed no pathological alterations. The large, prominent, hyalinized basophils had disappeared and the acidophils were packed with deep fuchsinophilic granules. The percentages of the various cell types were well within the normal range (Table 1).

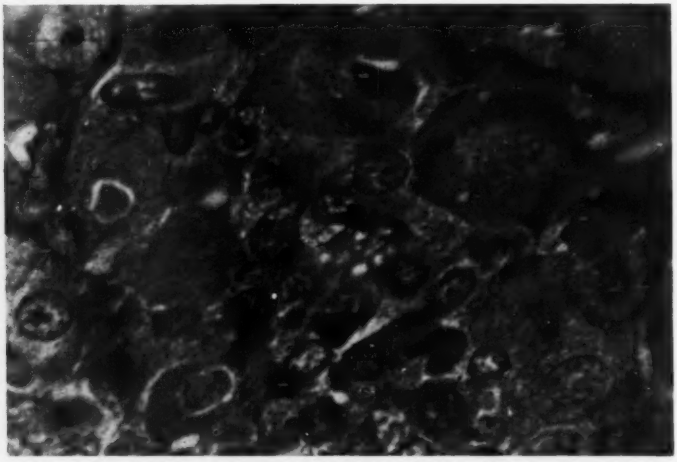

FIG. 3. Anterior pituitary gland from rat sacrificed eight months after a single injection of 875 microcuries I^{131}. Acidophils are degranulated, vacuolated basophils have regressed, and many large granular basophils remain. Martins; ×1200.

In contast to the normal picture presented by the pituitaries of animals which had received 300 microcuries were the glands of animals that had received 525 and 875 microcuries of I^{131} (fig. 3). In the latter, the acidophils were almost completely absent (Table 1), an occasional one being found only after careful search.

Five to eight months after the injection of 525 or 875 microcuries, the basophils had decreased in size and number as compared with those from animals sacrificed one month after treatment with the same amounts of radioiodine (Table 1).[4] In some animals, the hyalinized, vacuolated basophils were quite rare and in such rats the smaller,

[4] After reaching a value of about 30 per cent, the percentages of pituitary basophils in rats injected with 525 or 875 microcuries of I^{131} decrease. At the latest interval studied, namely 11 months after the injection of these I^{131} doses, the percentages of basophils found were in the neighborhood of 10 per cent.

A similar phenomenon has been observed by Griesbach after long-term treatment of rats with thiouracil (personal communication). This has also been observed in this laboratory.

non-vacuolated, granular type basophil predominated. In many animals, however, numerous hyalinized basophils were present in considerable number (fig. 3).

Two animals that had been injected with 525 microcuries of I^{131} showed no degranulation of the acidophils and only a few signet-ring cells. Serial section of the neck region showed these animals to have thyroid tissue of a hyperplastic, hypertrophic type resembling that seen after short-term treatment with propylthiouracil (Goldberg *et al.*, preceding paper).

B. THE PITUITARY WEIGHTS OF RATS INJECTED WITH I^{131}

Sixteen male rats each weighing 250 gm. were injected with 875 microcuries of I^{131}, a dose that completely destroys the thyroid gland (Goldberg *et al.*, preceding paper). These rats and their respective litter mate controls were sacrificed 11 months later. Their pituitaries were rapidly excised and fixed as described above. To avoid cellular distortion, the pituitary gland was weighed after complete fixation and initial dehydration in 70 per cent alcohol.

TABLE 2. DISTRIBUTION OF ACIDOPHILS, BASOPHILS, AND CHROMOPHOBES IN THE ANTERIOR PITUITARY GLANDS OF SURGICALLY THYROIDECTOMIZED RATS

Rat	Interval after thyroidectomy	Anterior pituitary		
		Acidophils	Basophils	Chromophobes
	months	per cent of total	per cent of total	per cent of total
1	1	1.4	28.4	70.2
2	1	1.0	30.2	68.8
3	1	0.8	23.7	75.5
4	1	1.2	29.4	69.4
5	1	0.9	33.6	65.5
6	6	1.0	14.9	84.1
7	6	0.8	21.3	77.9
8	6	0.9	16.4	82.7

Table 3 shows that the weights of the pituitary glands were slightly increased in the injected animal as compared with their controls. Several investigators have observed an increase in the weight of the rat pituitary gland after surgical excision of the thyroid gland (Griesbach and Purves, 1943; Zeckwer *et al.*, 1935; see also Table 3).

C. RESPONSE OF ANTERIOR PITUITARIES OF TOTALLY RADIOTHYROIDECTOMIZED RATS TO THYROXINE INJECTIONS

In the first communication, it was shown that a single injection of 3.5 millicuries of I^{131} per kg. body weight (875-microcurie dose) completely destroyed the rat thyroid. In rats so treated, serial sections of the entire cervical region revealed no vestige of thyroid epithelium (Goldberg *et al.*, preceding paper). The pituitaries of these animals underwent changes similar in all respects to those seen after surgical thyroidectomy, i.e., complete degranulation of the acidophils in

approximately three to four weeks, a marked increase in basophilic elements, and the presence of signet-ring (thyroidectomy) cells.

The experiments described below show that even as late as 11 months after complete destruction of the thyroids by I^{131}, the anterior pituitary still responds to injected thyroxine by acidophilic regranulation. In this respect, the similarity between radiothyroidectomy and surgical thyroidectomy is again demonstrated.

TABLE 3. WEIGHTS OF PITUITARY GLANDS OF MALE RATS SACRIFICED 11 MONTHS AFTER RADIO* AND SURGICAL THYROIDECTOMY

Treatment	Body weight	Weight of pituitary gland	Per cent body weight
	gm.	mg.	x 10^{-2}
Control	350	6.8	1.9
Control	365	7.4	2.1
Control	390	8.1	2.1
Control	410	9.1	2.2
Control	374	6.9	1.8
Control	358	7.8	2.2
875 Microcuries of I^{131}	380	9.0	2.4
875 Microcuries of I^{131}	350	6.3	1.8
875 Microcuries of I^{131}	342	9.3	2.7
875 Microcuries of I^{131}	374	11.4	3.0
875 Microcuries of I^{131}	324	7.8	2.4
875 Microcuries of I^{131}	254	11.4	4.5
875 Microcuries of I^{131}	350	11.8	3.4
875 Microcuries of I^{131}	325	10.2	3.1
875 Microcuries of I^{131}	330	8.1	2.5
875 Microcuries of I^{131}	350	7.2	2.1
875 Microcuries of I^{131}	310	8.0	2.6
875 Microcuries of I^{131}	420	11.3	2.7
875 Microcuries of I^{131}	380	9.3	2.5
875 Microcuries of I^{131}	390	9.0	2.3
875 Microcuries of I^{131}	360	9.7	2.7
875 Microcuries of I^{131}	370	7.9	2.1
Surgical Thyroidectomy	344	10.4	3.0
Surgical Thyroidectomy	278	11.3	4.1

* Rats were given a single injection of 875 microcuries I^{131}. They weighed approximately 250 gm. at time of injection.

One group of animals which had received a single injection of 875 microcuries of I^{131} was given daily intraperitoneal injections of 10 gamma of dl-thyroxine in isotonic saline for one month. Injections were begun 11 months after radiothyroidectomy, and continued for four weeks. Another group of surgically thyroidectomized rats was treated in the same manner. A third group of radiothyroidectomized rats was injected with isotonic saline only. The results are recorded in Table 4.

The injections of dl-thyroxine in the thyroidectomized rats (surgical and radio-) resulted in regranulation of the acidophils and a reduction in the percentages of the basophils of the pituitaries. The vacuo-

TABLE 4. CONDITIONS OF PITUITARIES FROM RATS 11 MONTHS AFTER THYROIDECTOMY BY BOTH SURGERY AND RADIOACTIVE IODINE. THE EFFECT OF THYROXINE INJECTION BEGUN AT THIS INTERVAL

Animal	Body wt.	Experimental treatment	dl-Thyroxine injected			Anterior pituitary cell counts			Remarks
			Total amount daily	Amount per 100 gm. body weight daily	Duration	Acidophils	Basophils	Chromophobes	
	gm.		gamma	gamma	days	per cent of total	per cent of total	per cent of total	
1	344	Surgical thyroidectomy	10	2.9	30	40.3	8.7	50.0	Many signet-ring cells and small granular basophils. Acidophils well granulated.
2	278	Surgical thyroidectomy	10	3.6	30	47.6	4.3	48.1	Same as 1.
3	350	875 microcuries I^{131}	0	—	—	0.1	8.4	91.5	Most basophils are of signet ring type. Some large granular basophils. Poorly granulated acidophils very rare.
4	254	875 microcuries I^{131}	0	—	—	0.1	9.2	90.7	Same as 3.
5	380	875 microcuries I^{131}	10	2.6	30	53.4	1.8	44.8	Vacuolated basophils very rare. Well granulated acidophils.
6	374	875 microcuries I^{131}	10	2.7	30	49.1	2.9	47.0	Both vacuolated and small granular basophils. Acidophils well granulated.
7	342	875 microcuries I^{131}	10	2.9	30	51.6	3.7	44.7	Majority of basophils are of the vacuolated type. Acidophils well granulated.
8	350	875 microcuries I^{131}	10	2.9	30	57.3	8.4	44.3	Same as 5.
9	324	875 microcuries I^{131}	10	3.1	30	54.8	3.2	41.0	Same as 5.

TABLE 5. SUMMARY OF CONDITION OF THYROID AND ANTERIOR PITUITARY OF RATS INJECTED WITH 18, 300, 525, AND 875 MICROCURIES OF I^{131}

I^{131} injected	Interval sacrificed after injection	Anterior pituitary cell counts (average values)				Condition of thyroid gland*
		Acidophils	Basophils	Chromophobes	Total cells counted	
microcuries		per cent of total	per cent of total	per cent of total		
None	Controls	43.2	5.1	51.7	43,000	Normal
875	6 hours	42.1	4.3	53.6	2,000	Normal
875	12 hours	44.7	5.7	49.6	2,000	Swelling and vacuolation of cytoplasm of epithelial cells, some degenerative nuclear changes.
875	24 hours	44.2	6.2	49.6	4,000	Glandular edema, inflammation, follicles contain very vacuolated colloid; swollen, vacuolated and pyknotic epithelial cells. Follicular disintegration.
875	72 hours	43.4	5.7	51.0	4,000	Almost complete glandular disorganization, few thyroid cells present, almost no colloid. Acute inflammation.
875	1 week	32.8	12.9	54.3	2,000	Follicular "ghosts," only a few degenerating thyroid cells.
875	2 weeks	14.3	22.5	63.3	4,000	Fibroblastic proliferation, a few thyroid epithelial cells visible. Mild chronic inflammation.
875	3 weeks	0.7	29.6	69.8	4,000	Thyroid area largely a fibrinous mass; fibrous organization.
875	4 weeks	0.9	25.2	73.9	4,000	Similar to three weeks.
875	6 months	0.9	18.0	81.4	6,000	No thyroid epithelium. Thin hyaline fibrous band with numerous pigment deposits.
875	8 months	1.0	19.4	79.9	8,000	Same as six months.
525	6 months	1.2	13.6	85.2	8,000	Thin fibrous band containing numerous atypical thyroid cells with little follicular organization, some colloid.
525	8 months	1.2	17.1	81.8	8,000	Similar to six months except greater numbers of the atypical epithelial cells.
525†	8 months	41.4	11.1	46.9	4,000	Hypertrophic thyroid epithelium with follicular organization.
300	1 week	37.6	8.9	53.1	4,000	Inflammation and edema decreased from that of 48 and 72 hours. New fibrous tissue being laid down.
300	2 weeks	27.0	11.7	60.8	4,000	Many intact typical follicles, fibrous proliferation, mild chronic inflammation.
300	3 weeks	27.8	13.9	58.4	4,000	Similar to two weeks.
300	4 weeks	44.7	17.2	38.2	4,000	Similar to two weeks, almost no sign of inflammation.
300	5–8 months	41.3	5.6	53.1	14,000	Normal small thyroids with increased interstitial fibrous tissue.
18	12 hours to 1 month	43.4	5.6	51.0	16,000	Normal.

* From previous communication (6).
† See text.

lated basophils also partially disappeared. It should be noted that the percentages of the various cell types in the thyroxine-treated rats were not normal (see Table 1) but resembled those observed in normal animals treated with thyroxine (Goldberg, unpublished observations). This might indicate that the amounts of thyroxine injected per day were in slight excess of the daily requirement.

DISCUSSION

Griesbach *et al.* (1949) have demonstrated that the basophils are not increased in the goitrogen-treated rat when 1.5 gamma l-thyroxine per 100 gm. body weight are injected daily. But when 1.4 gamma l-thyroxine per 100 gm. are injected, a significant increase in the number of basophils is seen. Thus a deficiency of 0.1 gamma l-thyroxine per 100 gm. body weight, per day, results in an increase in the percentage of basophils. The lack of a basophilic response in rats treated with 18 microcuries of I^{131} is, therefore, good evidence for the view that this dose does not impair the synthesis and secretion of normal amounts of thyroid hormone.

This conclusion regarding the effect of a single injection of 18 microcuries of carrier-free I^{131}, derived from a cytological study of the *anterior pituitary*, is in consonance with the evidence obtained by two other methods of study. In the preceding paper, it was shown that a single injection of 18 microcuries of I^{131} resulted in no observable change in the microscopic structure of the *thyroid* of the adult rat (Table 5). This dose of I^{131}, moreover, did not interfere with the specialized function of the thyroid in iodine metabolism, namely (1) iodine concentrating capacity, (2) iodine retention, and (3) the concentration of the circulating thyroid hormone, i.e., plasma protein-bound iodine (Feller *et al.*, 1949).

On the basis of these several pieces of evidence, it is reasonable to conclude that 18 microcuries of I^{131} may be safely employed as a tracer for metabolic studies in the adult rat.

With the injection of larger doses of I^{131}, the question of specific radiation damage to the anterior pituitary must be considered, particularly in view of Gorbman's (1949) recent observation of greatly enlarged pituitaries (as much as 100-fold increase in weight) in mice injected with 100–1000 microcuries of I^{131}. We wish to emphasize that in rats injected with 875 microcuries of I^{131} (the largest dose used in this study), the pituitaries were not larger than those observed in rats after surgical excision of their thyroids. Furthermore, no histological evidence of radiation damage in the pituitary (vascular changes, inflammatory reaction, etc.) was observed. Finally, the cytological changes, quantitative as well as qualitative, occurring in the pituitary glands of rats in which the thyroid gland was completely destroyed by 875 microcuries of I^{131} are identical with those observed

* From previous communication (6).
† See text.

both in this laboratory and elsewhere (Griesbach, 1941; Zeckwer *et al.*, 1935; Lebedewa, 1936; Reese *et al.*, 1943; Severinghaus *et al.*, 1933; and Hohlweg and Junkmann, 1933), after total surgical thyroidectomy (Table 2). These morphological findings leave little doubt that the changes found in the anterior pituitary of rats injected with 875 microcuries of I^{131} are solely the result of total thyroid destruction and not of a direct radiation effect.

To obtain further evidence on the condition of pituitaries of rats injected with 875 microcuries of I^{131}, we tested their capacity to respond to injected thyroxine. As late as 11 months after total destruction of the thyroid gland by the injection of 875 microcuries of radioactive iodine, the pituitaries still reacted to injected thyroxine in a manner similar in all respects to the pituitaries of rats surgically thyroidectomized, i.e., by regranulation of the acidophils and by pronounced regression[5] of the basophils. It may therefore be concluded that a study of the anterior pituitaries of rats injected with the doses of I^{131} used here is still valid as an indicator of thyroid function.

The major purpose of the present study was to secure information on the functional activity of the thyroid glands partially destroyed by the ionizing effects of radiation. Complete destruction of the thyroid gland was observed in rats which had received single injections of 875 microcuries of I^{131} (Table 5). Partial destruction of the thyroid gland occurred in rats treated with 525 and 300 microcuries (Table 5).

Five to eight months after single injections of 525 microcuries of I^{131}, many thyroid cells were observed in serial sections prepared from the cervical region. These were large, atypical cells containing hyperchromatic and pleomorphic nuclei, and granular eosinophilic cytoplasm. These cells resembled the Hürthle cells seen in some pathological thyroids of man. Although a few small follicles, rimmed by three or four atrophic cells and containing some colloid-like material, were found, the large, atypical, epithelial cells usually occurred in groups or cords (Goldberg *et al.*, preceding paper).

The anterior pituitaries of these rats (525 microcuries of I^{131}) were indistinguishable from those of *totally thyroidectomized rats* (875 microcuries of I^{131}). This finding indicates that these cells were not producing hormone, or, at least, not enough to regranulate the acidophils of the pituitary. In a recent communication, Lennox (1948) reported no chemical or histological evidence for function in similar appearing cells (Hürthle or Askanazy cells) in man. A study is now being made to determine what course these cells will take at longer periods after radioiodine injection.

Two of the 18 animals that received a single injection of 525 microcuries of I^{131} did not contain thyroids with the atypical structure (Tables 1 and 5). Instead, a hypertrophic gland with follicular arrange-

[5] See footnote 4.

ment was observed, a condition similar to that seen in normal rats treated for a short period with a chemical goitrogen such as propylthiouracil. The percentages of basophils in the pituitaries of these rats were higher than usual, and the percentages of acidophils were normal (Table 1)—findings that indicate a slight thyroxine deficiency (Table 5).

After the injection of 300 microcuries of I^{131}, a large portion of the thyroid gland was destroyed. Five to eight months later, these thryoids were small but of normal histological appearance (Goldberg *et al.*, preceding paper) (Table 5). Examination of their pituitaries revealed that these animals had a thyroxine deficiency for at least one month after the I^{131} injection. Five to eight months later, however, the pituitary glands gave no indication of thyroxine deficiency as judged by the percentage of basophils.

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SUMMARY

A cytological study of the pituitaries of rats injected with 18, 300, 525, and 875 microcuries of I^{131} is presented. The findings have been used in evaluating the extent of thyroid function in the animals.

The injection of 18 microcuries of I^{131} resulted in no cytological changes in the anterior pituitary.

From several lines of evidence (thyroid histology, pituitary cytology, and metabolic functions of the thyroid gland), the conclusion is drawn that a single injection of 18 microcuries of I^{131} can be safely used as a tracer for metabolic studies in the adult rat.

Evidence is presented to show that the changes occurring in the anterior pituitaries of rats after the injection of large amounts of I^{131} are not the result of radiation damage to the pituitary itself but solely the consequence of thyroid destruction.

The injection of 300 microcuries of I^{131} resulted in considerable thyroid destruction, but five to eight months later, normal glandular function was regained as judged by a study of the anterior pituitary.

It is shown here, from a study of the reactions in the anterior pituitary that the atypical thyroids present 6–8 months after single injections of 525 microcuries of I^{131} synthesize negligible amounts of thyroxine or none at all.

The cytological changes occurring in the anterior pituitary after the injection of 875 microcuries of I^{131} are identical with those occurring after total surgical thyroidectomy.

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METABOLISM OF THE STEROID HORMONES: METABOLISM OF DEHYDRO-ISOANDROSTERONE[1]

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THE METABOLISM of dehydroisoandrosterone was studied in 2 individuals, a normal woman and a man with Addison's disease. While this work was in progress, Mason and Kepler (1945, 1947) reported the results of administration of dehydroisoandrosterone to 3 patients. Our findings confirm those of these workers, that androsterone and etiocholanol-3(α)-one-17 were the major products obtained from dehydroisoandrosterone. These 2 compounds are also the major products obtained after administration of testosterone (Dorfman *et al.*, 1939; Callow, 1939).

Following administration of dehydroisoandrosterone to the patient with Addison's disease, etiocholanediol-3(α),17(α) was also isolated, and has been previously reported (Miller and Dorfman, 1948); up to this time it had not been found to occur in human urine.

EXPERIMENTAL

Melting points were determined with the Fisher-Johns apparatus and are uncorrected. 17-Ketosteroids were determined by the Holtorff-Koch (1940) method.

Patient 1 (E.H.)

A normal woman received 1800 mg. dehydrosisoandrosterone acetate* (equivalent to 1570 mg. dehydroisoandrosterone), injected intramuscularly in doses of 150 mg. in 3 cc. sesame oil twice daily for 6 days. The urine was collected for 5 of the 6 days during the injection period, and for 2 days after the injections were discontinued. The total excretion of 17-ketosteroids was 474 mg.

Hydrolysis and Extraction. The procedures used are shown in

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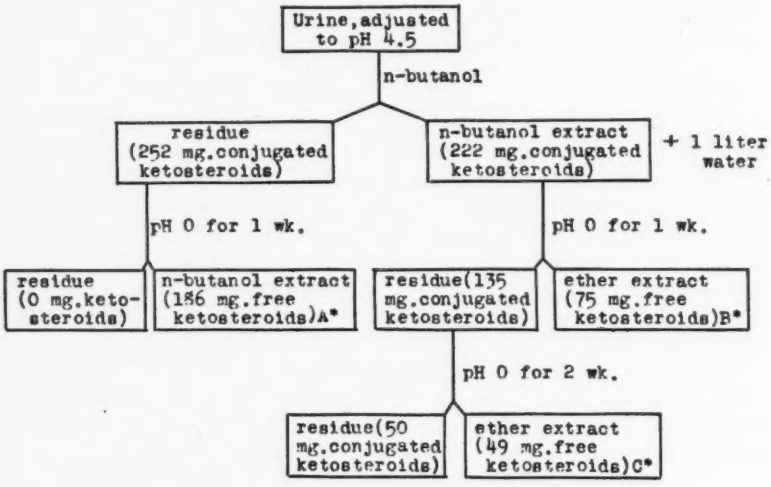
* The dehydroisoandrosterone was the generous gift of Ciba Pharmaceutical Products, Inc.

Figure 1. From this figure it can be seen that of the 474 mg. 17-ketosteroids originally present, 310 mg., or 65% ,were obtained in the free form, and an additional 50 mg. left in the residual urine in conjugated form. Since n-butanol extracts both the free and water soluble conjugated steroids, whereas ether extracts only the free steroids, it was necessary to determine if the ketosteroid values of the n-butanol extracts represented free or conjugated ketosteroids. Therefore,


Urine, adjusted to pH 4.5
n-butanol
residue (252 mg. conjugated ketosteroids)
n-butanol extract (222 mg. conjugated ketosteroids)
+ 1 liter water
pH 0 for 1 wk.
pH 0 for 1 wk.
residue (0 mg. keto-steroids)
n-butanol extract (186 mg. free ketosteroids)A*
residue (135 mg. conjugated ketosteroids)
ether extract (75 mg. free ketosteroids)B*
pH 0 for 2 wk.
residue (50 mg. conjugated ketosteroids)
ether extract (49 mg. free ketosteroids)C*

*A, B and C were combined.

FIG. 1. Hydrolysis and extraction at room temperature of urine of patient E. H.

aliquots of the n-butanol extracts were distributed between ether and water, and the material from each solvent assayed for 17-ketosteroids.

Fractionation. The 3 fractions (A, B and C) containing free steroids (Fig. 1) were combined and separated into ketonic and non-ketonic fractions with Girard's reagent T. Each of the 2 fractions was separated into α and β fractions with digitonin (1% solution in 90% ethanol), and the resulting 4 fractions chromatographed on aluminum oxide. The fractions eluted were unusually impure, due to the large amount of material extracted with n-butanol at low pH.

Ketonic Fractions. The α ketonic fraction (371 mg.) was dissolved in 5 cc. carbon tetrachloride, adsorbed on 12 gm. aluminum oxide and eluted with 50 cc. portions of carbon tetrachloride containing increasing amounts of ethanol.

Androsterone. About 30 mg. androsterone were obtained. The melting point was 172–178°; when mixed with an authentic sample

m.p. 180–182°, the melting point was 174–179°. An acetate was prepared, m.p. 162–163.5°; when mixed with androsterone acetate, m.p. 163–164°, the melting point was 162–164°.

Etiocholanol-3(α)-one-17. About 50 mg. etiocholanol-3(α)-one-17 were obtained. The melting point was 134–137° and 143–147°; when mixed with an authentic sample, m.p. 137–138° and 147–148°, the melting point was 135–138° and 146–147°. An acetate was prepared, m.p. 92–95°; when mixed with etiocholanol-3(α)-one-17 acetate, m.p. 96–96.5°, the melting point was 94–96°.

The β ketonic fraction (22 mg.) was acetylated (24.6 mg.), dissolved in 5 cc. benzene-petroleum ether 1:4, adsorbed on 1.5 gm. aluminum oxide and eluted with 10 cc. portions of benzene-petroleum ether 1:4, 2:3, 3:2, benzene, benzene-ether and ether.

Dehydroisoandrosterone. About 7 mg. dehydroisoandrosterone acetate, m.p. 157–164°, were obtained. When mixed with an authentic sample, m.p. 163–165°, the melting point was 160–164°. The saponified material yielded crystals, m.p. 136–149°; when mixed with dehydroisoandrosterone, m.p. 149–150°, the melting point was 145–150°.

Non-ketonic Fractions. The non-ketonic α fraction (507 mg.) was acetylated (530 mg.), dissolved in 6 cc. benzene-petroleum ether 1:1, adsorbed on 15 gm. aluminum oxide and eluted with 50 cc. portions of benzene-petroleum ether 1:1, 3:1, benzene, benzene-ether, ether and ether-ethanol.

Δ^5-Androstenediol-3(β),17(α). Elution with benzene-petroleum ether 1:1 yielded 35 mg. crystalline material which was rechromatographed. Elution with benzene-petroleum ether 1:1 yielded 28 mg. crystalline material of low melting point. Repeated crystallization yielded about 4 mg. Δ^5-androstenediol-3(β), 17(α) diacetate, m.p. 154.5–158°; when mixed with an authentic sample, m.p. 156.5–158°, the melting point was 152–155°. The material was saponified, and after recrystallization from aqueous acetone, the melting point was 177–179°. When mixed with Δ^5-androstenediol-3(β),17(α), m.p. 179–182°, the melting point was 178–180.5°. The occurrence of this β compound in the α fraction is apparently due to the relatively soluble digitonide which it forms.

The non-ketonic β fraction (17.4 mg.) was acetylated (19.4 mg.), dissolved in 6 cc. benzene-petroleum ether 1:2, and adsorbed on 1 gm. aluminum oxide.

Cholesterol. Elution with 20 cc. of the solvent yielded about 12 mg. cholesterol acetate, m.p. 113–114°. When mixed with an authentic sample, m.p. 112.5–113.5°, the melting point was 112.5–114°.

Patient 2 (F.S.)

A man with Addison's disease received 1350 mg. dehydroisoandrosterone by mouth in doses of 150 mg. each, 3 times daily for 3 days. The urine was collected for 2½ days during the administration

period. The sum of the daily 17-ketosteroid determinations indicated a total value of 326 mg.

Hydrolysis and Extraction. The urine was acidified with 15% concentrated hydrochloric acid (by volume) and refluxed for 15 minutes. After addition of 10 gm. sodium chloride per 100 cc., the hydrolyzed urine was extracted with ¼ volume benzene 3 times, the benzene evaporated, the material taken up in ether, washed with alkali and water, and the ether evaporated, yielding the neutral fraction.

Fractionation. The neutral fraction (0.3 gm.) was separated into ketones and non-ketones with Girard's reagent T. The ketonic fraction was further fractionated with digitonin. These 3 fractions were chromatographed on aluminum oxide.

Ketonic Fractions. The α fraction (207 mg. originally, 199 mg. after loss in handling) was dissolved in 6 cc. benzene, adsorbed on 6 gm. aluminum oxide and eluted with 25 cc. portions of benzene containing increasing amounts of ethanol. *$\Delta^{2\text{and}3}$-Androstenone-17.* About 10 mg. $\Delta^{2\text{and}3}$-androstenone-17 were obtained, m.p. 97-104°; when mixed with an authentic sample, m.p. 105–106.5°, the melting point was 101–105°. *Androsterone.* About 20 mg. androsterone were obtained, m.p. 178–183°; when mixed with an authentic sample, m.p. 180–183°, the melting point was 179–183°. An acetate was prepared, m.p. 163–164°; when mixed with androsterone acetate, m.p. 163–164°, the melting point was 162–164°. *Etiocholanol-3(α)-one-17.* About 18 mg. etiocholanol-3(α)-one-17 were obtained, m.p. 135° and 146°; when mixed with an authentic sample, m.p. 141–142° and 149.5–151.5°, the melting point was 137–140° and 149–150°. An acetate was prepared, m.p. 89–94°; when mixed with etiocholanol-3(α)-one-17 acetate, m.p. 96–96.5°, the melting point was 91–95°.

The β ketonic fraction (5 mg.) yielded no sharply melting material.

Non-ketonic Fraction. This fraction (33.6 mg.) was acetylated (38.2 mg.), dissolved in 2 cc. benzene-petroleum ether 1:3, adsorbed on 3 gm. aluminum oxide and eluted with 20 cc. portions of benzene-petroleum ether 1:9, 1:4, 1:2, 3:1, benzene, benzene-ether, ether and ether-ethanol. *Etiocholanediol-3(α),17(α).* Elution with benzene-petroleum ether 1:1 yielded about 5 mg. etiocholanediol-3(α),17(α) diacetate, m.p. 126–126.5°. When mixed with an authentic sample, m.p. 126–127°, prepared by reduction of etiocholanol-3(α)-one-17 with sodium and isopropyl alcohol and subsequent acetylation, the melting point was 126–127°. *Compound, m.p. 174°-176.* Elution with ether yielded a small amount of a compound, m.p. 174–176°, which when mixed with Δ^5-androstenediol-3(β),17(α), m.p. 180–181°, melted at 123–150°. This compound, m.p. 174–176°, together with the mother liquors from the etiocholanediol-3(α),17(α) diacetate were rechromatographed, yielding only etiocholanediol-3(α),17(α) diacetate, m.p.

124–127°. It is possible that this compound is etiocholanediol-3(α),17(α) monoacetate.

A summary of the compounds isolated is given in Table 1.

TABLE 1. SUBSTANCES ISOLATED FROM URINE AFTER ADMINISTRATION OF DEHYDROISOANDROSTERONE

Compound	Patient 1 (mg.)	Patient 2 (mg.)
$\Delta^{2 \text{ and } 3}$-Androstenone-17		10
Androsterone	20	20
Etiocholanol-3(α)-one-17	50	18
Dehydroisoandrosterone	7	
Δ^{5}-Androstenediol-3(β),17(α)	4	
Etiocholanediol-3(α),17(α)		5
Cholesterol	12	

DISCUSSION

In view of the high excretion of β-ketosteroids reported to occur in adrenal cortical carcinoma (Talbot *et al.*, 1940a, 1940b, 1942; Wolfe *et al.*, 1941; Dingemanse *et al.*, 1946; Kepler and Mason, 1946; Johnson and Nesbit, 1947; Hirschmann and Hirschmann, 1947; Miller and Dorfman, 1947; Dobriner *et al.*, 1948), it was of interest to determine the further pathways of dehydroisoandrosterone in the absence of a malignant cortex. It is apparent from our results, summarized in Table 1, and those of Mason and Kepler (1947), that dehydroisoandrosterone is metabolized chiefly to unknown products, and that the major metabolites recovered are androsterone and etiocholanol-3(α)-one-17. Nevertheless, an increased excretion of androsterone has not been reported to accompany the excessive amounts of dehydroisoandrosterone excreted in adrenal cortical carcinoma. The capacity for disposing of large amounts of dehydroisoandrosterone is shown by the small recovereis of unchanged dehydroisoandrosterone. As Mason and Kepler (1947) have pointed out, this compound, therefore, is not necessarily an endproduct of steroid metabolism. It is of interest that the one patient who showed an appreciable excretion of dehydroisoandrosterone following its administration (7% recovery of unchanged material) was suffering from acute anterior pituitary insufficiency (Mason and Kepler, 1947) in contrast to the low figures obtained in 1 normal patient (0.5% recovery) and in 3 patients with Addison's disease (0 recovery), 2 of which were reported by Mason and Kepler. In these 5 cases, more androsterone then etiocholanol-3(α)-one-17 was isolated from urine of the 3 male patients, whereas more etiocholanol-3(α)-one-17 than androsterone was isolated from the female patients. It is not apparent whether this represents a sex difference in the metabolism of dehydroisoandrosterone, or merely individual variations.

SUMMARY

Following the administration of dehydroisoandrosterone to a normal woman and to a man with Addison's disease, androsterone and etiocholanol-3(α)-one-17 were recovered as the major metabolites. The amounts recovered by isolation (minimal values) indicated a conversion of 1% and 2%, respectively, to androsterone, and 3% and 1%, respectively, to etiocholanol-3(α)-one-17. Recovery of unchanged dehydroisoandrosterone was 0.5% and 0, respectively. Δ^5-Androstenediol-3(β),17(α), etiocholanediol-3(α),17(α) and cholesterol were also isolated. The organism is able to further metabolize large amounts of dehydroisoandrosterone, chiefly to unidentified products.

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THE EFFECTS OF STEROIDS ON THE INCIDENCE OF DIABETES IN RATS AFTER SUBTOTAL PANCREATECTOMY

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SEVERAL facts have shown the gonads and sexual hormones exert an influence on the development of diabetes in pancreatectomized rats: (1) the incidence of diabetes after removal of 95 per cent of the pancreas is greater in male than in female rats (Foglia, 1945); (2) castration markedly increases diabetes in females and slightly decreases it in males; (3) treatment with estradiol benzoate diminishes considerably the incidence of diabetes in intact and castrated male and female rats following removal of 95 per cent of the pancreas (Foglia, Schuster and Rodríguez, 1947).

There are contradictory reports on the effect of estrogens on diabetes in other species. Thus, according to one group of workers the severity of diabetes is diminished by treatment with estrogens in the dog (Barnes, Regan and Nelson, 1933) and in the monkey (Nelson, 1935; Nelson and Overholser, 1936). On the other hand, certain workers have not observed any effects in the dog (Young, 1941) or in the monkey (Collip, Selye and Neufeld, 1937). In the ferret, estrogens have been reported to increase the severity of diabetes (Dolin, Joseph and Gaunt, 1941). In certain conditions stilbestrol provokes glycosuria in normal and subtotally pancreatectomized rats (Ingle, 1941 and 1947; Ingle and Nezamis, 1943), but in subtotally pancreatectomized rats, under forced feeding and stilbestrol treatment after 4 to 5 weeks the blood sugar returns to normal level and remains normal in spite of the injections; meanwhile the noninjected controls develop diabetes (Rodríguez, unpublished observations).

The general opinion seems to be that sexual hormones have little or no effect on diabetes, but Ingle's work has led to the belief that estrogens increase the severity of the disease. Previous work on diabetes in the rat done in this laboratory, and the observations reported in this paper show that sexual hormones have a definite effect on the incidence and severity of diabetes in the subtotally (95 per cent) pancreatectomized rat.

METHODS

White rats from a pure bred strain were castrated when they had reached 80 to 100 g. body weight. A week later 95% of the pancreas was removed

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following Foglia's (1944) technique. Each lot was operated by the same person at the same time, and was afterwards divided into groups receiving treatment with one or other steroid, and controls injected with lactose; thus differences due to removal of a varying amount of pancreas were reduced to a minimum. A week after pancreatectomy treatment was commenced, consisting in one daily subcutaneous injection of the steroid mixed with 25 mg. of lactose suspended in 0.5 ml. saline (0.9 % NaCl) solution. The controls received 25 mg. lactose in saline. The treatment was continued for six months. The animals were fed "ad libitum" a diet, the composition of which is given in Table I.

TABLE 1. COMPOSITION OF DIET

	Per cent	Carbohydrate	Protein	Fat	Water	Cellulose	Calories
Milk	64	3.07	1.98	1.98	55.68	—	38.80
White bread	20	11.48	1.85	0.03	6.17	0.04	53.70
Wheat	8	6.09	0.84	0.11	0.64	0.13	28.75
Corn	8	5.31	1.01	0.29	0.76	1.60	27.97
Total	100	25.95	5.68	2.41	63.25	1.77	149.22

One day per week 0.2 per cent brewer's yeast, 0.08 per cent cod liver oil and a small amount of fresh alfalfa was added.

The body weight and blood sugar were determined monthly, and an animal was considered to be diabetic when the blood sugar was above 150 mg. per cent after a 7 hour fast. At the end of the experiment the animals were killed, the organs weighed and the pancreatic tissue remaining was fixed in Helly and Bouin-Holland solutions. Paraffin sections of 4 μg. were made and stained with hematoxylin-eosin, Gomori's chromic hematoxylin and Heidenhein's azocarmine, following Gomori's modification for the Langerhans islets.

RESULTS

The results of each series are given in Tables 2, 3, 4 and 5, and chart 1.

Estrone: This substance exerted a protective action in doses of 15 and 50 μg. daily, the effect being more marked with the larger dose. The hair on the caudal part of the back and on the abdomen was lost almost completely. There was a slight fall in body weight and in the weight of the thymus. The hypophysis, adrenals, thyroid, uterus and kidney were enlarged (Table 4).

Stilbestrol (dipropionate): Daily doses of 15 and 50 μg. had a protective action on castrates of both sexes, which was more marked with the higher dose. There was considerable loss of hair all over the body in most animals and a marked fall in body weight. The hypophysis was found to be greatly enlarged; the adrenals were much above the normal weight, and the uterus, kidneys and liver were also enlarged. The prostate and seminal vesicles were very large although the animals were castrated; in one case hypertrophy of the prostate caused retention of urine in the bladder and the animal died. The

TABLE 2. EFFECT OF DIFFERENT SUBSTANCES INJECTED DAILY DURING 6 MONTHS ON THE INCIDENCE OF DIABETES IN MALE AND FEMALE CASTRATED RATS, AFTER REMOVAL OF 95 PER CENT OF THE PANCREAS

Drug	Daily dose per rat μg.	Castrated females				Castrated males	
		Diabetics	Percentage	Diabetics	Percentage	Diabetics	Percentage
Lactose	25 (mg)	12/23	52	26/35	74	16/18	89
Cholesterol	500	4/15	27	5/9	55		
Estrone	15	3/10	30				
Estrone	50	0/8	0				
Stilbestrol	15	3/10	30				
Stilbestrol	50			1/8	12	3/6	50
Dienestrol	50			1/15	6		
Ethynil-estradiol	50			4/16	25		
Ethylnil-testosterone	500			4/9	44		
Equilenine	50	6/8	75				
Progesterone	500			6/9	66		
Desoxycorticosterone	100			8/12	66		
Testosterone	50	11/15	73				
Testosterone	500	8/8	100			13/14	92
Methyl-testosterone	500			9/11	83		
17-β-ethyl-dihydro-testosterone	500			8/11	72		

thyroid showed no change in weight and the thymus a moderate atrophy.

Dienestrol: Doses of 50 μg. daily had a protective effect. The general condition of the treated animals was good, although the body weight was slightly below that of the controls. There was also some fall of hair on the caudal part of the back and the abdomen. The hypophysis, adrenals, uterus, kidneys and liver were enlarged; the thyroid and thymus were of normal weight.

Estradiol (benzoate): This substance had a protective action when injected in doses of 2, 4 and 15 μg. in intact and castrated animals of both sexes. The results are summarized in Table 3.

TABLE 3. EFFECT OF ESTRADIOL BENZOATE INJECTED DAILY DURING 6 MONTHS ON THE INCIDENCE OF DIABETES IN INTACT AND CASTRATED MALE AND FEMALE RATS AFTER REMOVAL OF 95 PER CENT OF THE PANCREAS (FOGLIA, SCHUSTER AND RODRÍGUEZ)

Daily dose per rat μg.	Intact Males				Castrated Males			
	Controls		Treated		Controls		Treated	
	Diabetic	Percentage	Diabetic	Percentage	Diabetic	Percentage	Diabetic	Percentage
2	11/12	92	4/11	36	8/13	61	4/11	36
4	8/9	88	4/8	50	7/8	87	4/9	44
15	10/10	100	2/12	16	8/8	100	6/9	66
	Intact Females				Castrated Females			
2	6/14	43	1/12	8	2/9	22	2/12	16
4	13/63	21	—	—	19/52	37	1/13	8
15	5/7	71	2/6	33	6/7	86	1/8	12

TABLE 4. WEIGHT OF ORGANS (IN MG. PER 100 G. BODY WEIGHT) OF FEMALE CASTRATED RATS INJECTED DIFFERENT ESTROGENS DAILY DURING SIX MONTHS AFTER REMOVAL OF 95 PER CENT OF THE PANCREAS

Organ	Diab.	Lactose 25 mg.	Estrone		Stilbestrol		Dienestrol 50 μg.	Estradiol 15 μg.	Ethynil estradiol 50 μg.	Ethynil testosterone 500 μg.	Equilenine 50 μg.
			15 μg.	50 μg.	15 μg.	50 μg.					
Hypophysis	No	5.5	7.6	19.0	12.3	21.5	13.3	27.0	67.4	5.3	9.4
	Yes	6.1	7.0	—	—	—	—	—	—	5.1	9.0
Adrenals	No	18.7	35.0	38.4	41.7	42.8	47.4	36.0	50.9	18.6	36.2
	Yes	22.2	40.2	—	—	—	—	—	—	30.4	47.4
Thymus	No	82	108	99	80	63	71	120	62	112	77
	Yes	72	25	—	—	—	—	—	—	66	45
Thyroids	No	9.7	13.1	14.5	15.9	11.2	9.9	11.4	11.6	8.6	11.3
	Yes	9.6	9.7	—	—	—	—	—	—	10.3	13.2
Uterus	No	61	223	343	218	276	284	230	328	90	230
	Yes	74	304	—	—	—	—	—	—	118	240
Kidneys	No	802	982	1015	1009	1080	951	—	930	847	1100
	Yes	1146	1445	—	—	—	—	—	—	1218	1539
Liver	No	3404	—	—	—	5719	4076	—	4939	3969	—
	Yes	4503	—	—	—	—	—	—	—	5077	—
Body weight when killed (g)	No	212	193	180	197	175	177	—	166	208	186
	Yes	197	148	—	—	—	—	—	—	156	158

Ethynil-estradiol: A daily dose of 50 μg. decreased the incidence of diabetes. The body weight was much lower than that of the controls and there was considerable loss of hair; at the end of the six months' treatment the animals were almost completely bald. The enlargement of the hypophysis and adrenals was as great as in rats treated with stilbestrol. The uterus, liver and kidneys were also enlarged. The thymus and thyroid were of normal weight.

TABLE 5. EFFECT OF SEVERAL STEROIDS AND OTHER SUBSTANCES ON THE WEIGHT OF ORGANS (IN MG. PER 100 G. BODY WEIGHT) OF MALE AND FEMALE CASTRATED RATS WITH 95 PER CENT OF THE PANCREAS REMOVED

Organ	Diab.	Castrated females								Castrated males		
		Lactose 25 mg.	Cholester. 500 μg.	Testosterone		Methyltestosterone 500 μg.	17-β-ethyltestoster. 500 μg.	Desoxycorticost. 100 μg.	Progester. 500. μg.	Lactose 25 mg.	Testosterone 500 μg.	Stilbestrol 50 μg.
				50 μg.	500 μg.							
Hypophysis	No	5.5	5.9	3.8	—	4.3	4.0	6.2	6.4	4.6	4.4	26.3
	Yes	6.1	5.8	4.3	3.8	4.2	4.6	5.3	5.5	6.3	5.8	13.9
Adrenals	No	18.7	18.1	14.6	—	18.3	13.2	18.8	25.1	14.9	16.6	39.1
	Yes	22.2	24.2	18.3	17.1	20.3	17.3	19.1	27.1	31.9	30.4	57.2
Thymus	No	82	118	94	—	60	96	137	124	79	19	61
	Yes	72	61	61	38	45	84	87	73	50	29	36
Thyroid	No	9.7	8.2	10.7	—	9.0	8.6	8.6	13.2	10.1	12.2	10.5
	Yes	9.6	8.2	10.7	13.9	10.6	9.0	8.4	10.1	9.9	10.5	11.8
Uterus	No	61	80	42	—	204	80	95	99	—	—	—
	Yes	74	88	51	181	246	76	91	110	—	—	—
Kidneys	No	802	806	787	—	1256	861	903	983	749	1100	1034
	Yes	1146	1253	1010	1320	1518	1154	1003	1169	1510	1409	1813
Liver	No	3404	3807	4193	—	5014	3849	4072	4836	3774	4977	5763
	Yes	4503	4823	4212	—	5441	4826	4024	4846	6368	5309	7279
Body weight when killed (g)	No	212	209	218	—	172	204	180	176	257	180	161
	Yes	197	173	211	201	153	173	198	180	171	171	118

Ethynil-testosterone: This substance exerted a protective effect at doses of 500 μg. per day. The general condition of the animals was good and their body weight the same as that of the controls. There were no significant differences in the weight of the organs with respect to that of the controls.

Equilenine: There was no protective effect on the incidence and course of diabetes in animals treated with 50 μg. daily. There was some fall of hair and the body weight was slightly lower than that of the controls. The hypophysis, adrenals, uterus and kidneys were enlarged, the thymus atrophied, and the thyroid was of normal weight.

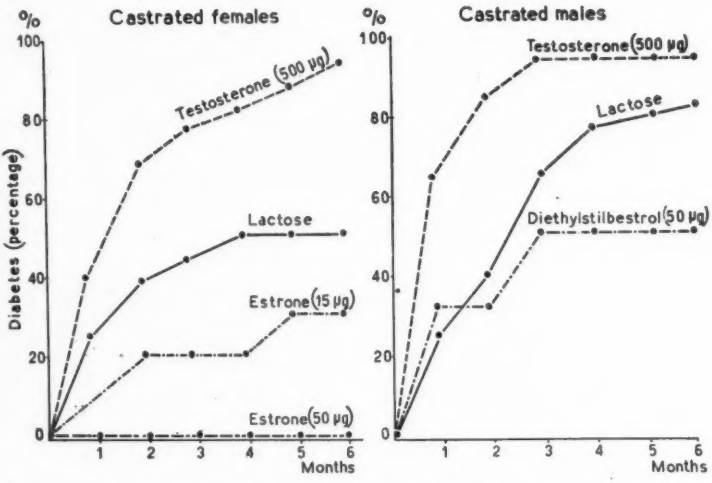

CHART 1. Incidence of diabetes in castrated female and castrated male rats, with subtotal pancreatectomy (removal of 95% of the pancreas) injected daily during six months with estrogens and androgens (controls with lactose); daily dose per rat in micrograms.

Progesterone: A dose of 500 μg. per day had no effect on the incidence of diabetes. The general condition was good, but the body weight was slightly lower than that of the controls. The weights of the hypophysis, thyroid and thymus were normal; the adrenals, uterus, liver and kidneys were slightly enlarged.

Desoxycorticosterone (acetate): Doses of 100 μg. per day had no effect on the incidence of diabetes. The general condition was good and the body weight was similar to that of the controls. Outside a slight enlargement of the uterus there were no significant variations in the weight of the organs.

Testosterone (propionate): Doses of 500 μg. daily increased the incidence and accelerated the onset of diabetes in both sexes. This was specially marked in the castrated males; at the end of the second

month 3 out of 15 (21 per cent) of the controls receiving lactose and 12 out of 14 (85 per cent) of those receiving testosterone were diabetic.

The increase in body weight of both sexes was below that observed in the controls, the difference being specially marked in the male castrates. The hair did not fall but became rough and dirty after 1 to 2 months' treatment. The males had the penis in continuous semi-erection. The liver, kidneys and genital organs (uterus in the female, prostate and seminal vesicles in the male) were enlarged; the thyroid was of normal weight, and the thymus was considerably atrophied; the hypophysis and adrenals weighed slightly less than in the controls.

Methyltestosterone: Daily doses of 500 μg. accelerated the onset of diabetes. The body weight was below that of the controls, and the general condition was poor, although not so bad as that of animals injected with testosterone. The hypophysis was slightly atrophied. The weights of the thyroid, thymus and adrenals were normal. The liver and kidneys were enlarged and the uterus was large, congested and filled with fluid.

17-β-ethyl-dihydro-testosterone: This substance had no effect on diabetes when given in doses of 500 μg. daily. The general condition was good and the body weight similar to that of the controls. The hypophysis and adrenal were below normal weight, the uterus slightly enlarged, and the thyroid, thymus, kidneys and liver normal.

Cholesterol: Doses of 500 μg. daily exerted a slight but definite protective effect (see Table 2). The body weight and the weight of the organs did not differ significantly from the controls.

Lactose: Controls were injected lactose and the results of each group are given in the tables.

The effects of diabetes: When the animals became diabetic they lost weight and eventually ended in cachexy. The general condition was specially poor when the drug injected exerted an unfavorable effect, as was the case with stilbestrol, ethynil-estradiol and testosterone. Complications of diabetes, such as cataract, were seen with equal frequency in diabetic animals treated with steroids as in the untreated controls.

The autopsy showed the hypophysis of diabetic animals in cachexy was smaller than that of nondiabetic animals in the same group; there was also atrophy of the thymus and enlargement of the adrenals, liver and kidney. There were no changes in the weight of the thyroid and uterus except in cachectic animals, in which these organs were atrophied.

Histological examination: The remaining pancreatic tissue was examined histologically by Dr. A. F. Cardeza. The controls treated with lactose showed sclerosis due to peri-insular growth of connective tissue with penetration between the islet cells and a variable degree

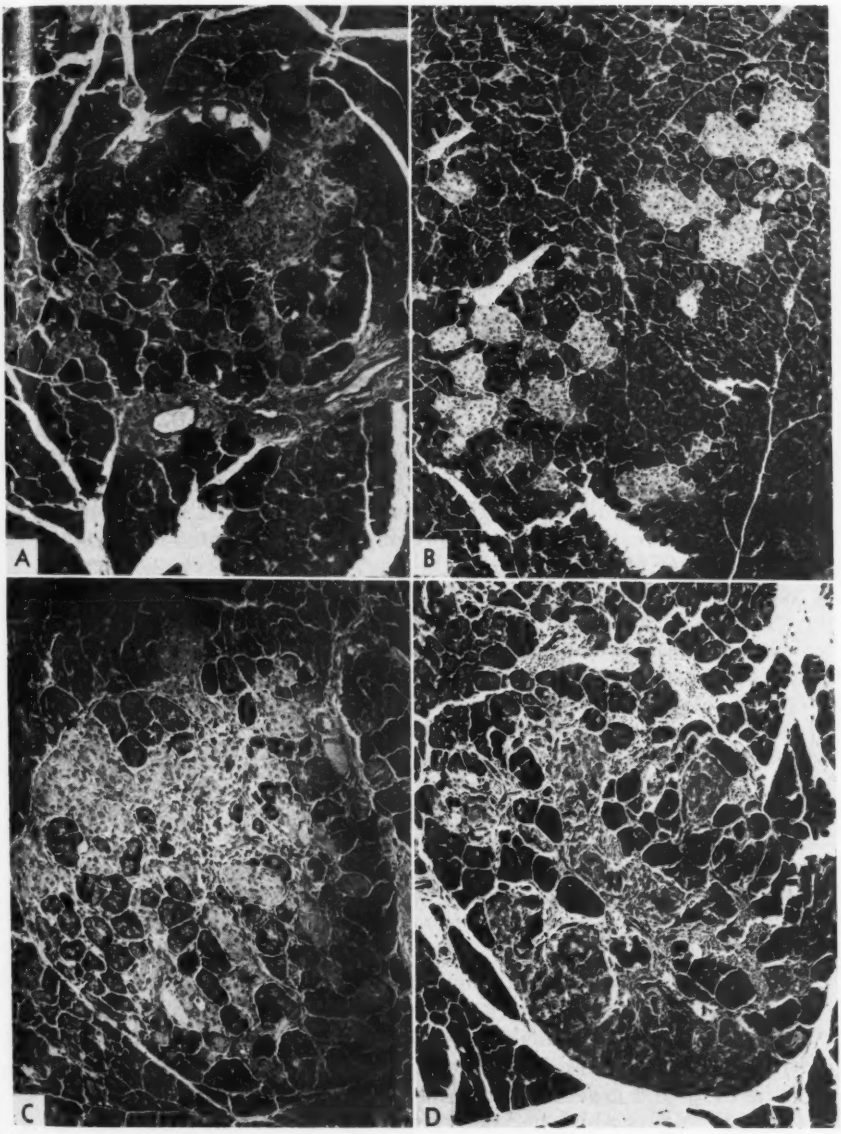

FIG. 1 Pancreas of nondiabetic partially pancreatectomized rats (removal of 95% of the pancreas), injected daily during 6 months with: A) lactose, showing sclerosis inside and outside the islets of Langerhans; B) ethynil-estradiol showing an increased number of islets; C) diethyl-stilbestrol showing marked hypertrophy and hyperplasia of the islets; D) diethyl-stilbestrol showing adenomatous proliferations of the islets of Langerhans. Hematoxylin-eosin ×80.

of insular atrophy (fig. 1-a). In the non-diabetic controls the β cells were diminished in number, but the granules are well conserved and stained normally with chromic hematoxylin. The pancreas of diabetic animals stained with the same technique showed several changes: the granules were displaced to the periphery of the β cell, or were absent, there was vacuolization of the protoplasm, and a few cells had pycnotic nuclei. In some of the islets no β cells were found.

Animals treated with cholesterol which did not become diabetic showed moderate insular hyperplasia, and small newly formed islets; the pancreas of diabetic animals in this series was in a similar condition to that of the diabetic controls.

Animals treated with estrone, estradiol, stilbestrol and ethynil-estradiol (fig. 1b, c and d) showed typical changes in the pancreas, consisting in marked hyperplasia of the insular tissue, with hypertrophy of the islets in some cases. In certain animals the newly formed islets occupied a whole pancreatic lobule, displacing the acini, which showed signs of damage due to compression. The aspect was that of an insular adenoma.

There was usually a single spherical or lobulated newly formed islet in a pancreatic lobule, with prolongations insinuated between the cells of the acini. In other cases the newly formed islets were gathered in clusters. There was little or no peri-insular collagenous reaction.

Frequently in the center of the acini groups of hypertrophied cells, similar to β cells, in contact with centro-acinous cells were found. In favorable sections it was possible to see the continuity between centro-acinous cells loaded with α granules and a newly formed islet, which was seen joined to the acinus by a pedicle of centro-acinal cells. These islets were formed mainly by β cells with a thin cortical layer of α cells, which sometimes was missing.

Rats which had been treated with estrogens but had become diabetic had moderate insular hyperplasia, with a marked decrease in β cells. These cells had lost their granules and showed vacuolization. Insular hyperplasia was greater in the animals treated with 50 μg. estrone daily than in those which had received only 15 μg. daily. There was no correlation between the degree of hyperplasia and the dose injected in rats treated with stilbestrol.

In rats treated with ethynil-estradiol and ethynil-testosterone, which had not become diabetic hyperplasia of the islet tissue was observed; while in those which became diabetic sclerosis and atrophy of the islets and degenerative lesions in the β cells were found.

Diabetic rats which had been treated with equilenine had sclerosis and atrophy of the islets.

Diabetic rats which had been injected testosterone and methyl-testosterone showed marked sclerosis and atrophy of the islets. There were only α cells with one or two degranulated and vacuolized β cells. In one animal treated with methyl-testosterone which had not become

diabetic there were a fair number of islets with some sclerosis but with well granulated β cells.

Rats treated with progesterone, desoxycorticosterone and 17-β-ethyl-dihydro-testosterone which had not become diabetic showed moderate insular hyperplasia with collagenous fibers surrounding the islets, i.d. incipient sclerosis. Diabetic rats in this group showed sclerosis and atrophy of the islets with typical lesions in the β cells.

DISCUSSION

The incidence of diabetes in castrated subtotally pancreatectomized rats was modified by the action of several substances. Nearly all the estrogens tested diminished the incidence of diabetes; the most efficient at the doses given, were estrone, stilbestrol, dienestrol and estradiol. Ethynil-estradiol and ethynil-testosterone were less active and equilenine had no effect. It should be remembered that ethynil-testosterone had estrogenic, androgenic and progestational activity.

Androgens, on the contrary, increased the incidence and accelerated the onset of diabetes. Testosterone was more potent in this respect than methyl-testosterone, the animals becoming diabetic two months after beginning the treatment.

Substances with progestational activity such as progesterone, corticoids (desoxycorticosterone) and 17-β-ethyl-dihydro-testosterone, had no effect on the course of diabetes in the castrated white rats.

These facts are in agreement with the observations of Foglia, Schuster and Rodríguez, showing the protective effect of the hormones secreted by the ovary, and the unfavorable effect of those secreted by the testes, since castration decreased the incidence and severity of diabetes in males and increased it in females, after the removal of 95 per cent of the pancreas.

Cholesterol had a slight, but definite protective action which is particularly interesting because vaginal smears showed this substance had no estrogenic effect in these animals, since they were in permanent diestrus. Moreover, it did not cause any change in the weight of the hypophysis or the adrenals, or in body weight. Nevertheless histological examination showed hyperplasia of the pancreatic islets, although less marked than that produced by estrogens.

The mechanism by which estrogens and androgens exert their influence is still unknown. There are three possibilities: (a) the hormones act through the hypophysis, or the adrenals, or on the liver; (b) they act directly on the pancreas; or (c) they act on peripheral tissues, e.g. the muscle fiber.

Animals treated with stilbestrol, estrone, ethynil-estradiol and testosterone which had not become diabetic weighed less than the controls; the possibility of the influence of spontaneous restriction in the intake of food in these cases is being examined by means of forced feeding experiments.

Substances which have a protective effect against diabetes cause enlargement of the hypophysis, in some cases (estrone) in proportion with the dose given. There is, however, no strict correlation between the increase in hypophyseal weight and the protective action; thus ethynil-testosterone protects against diabetes but does not enlarge the hypophysis, while equilenine which has no protective effect causes an increase in the weight of the hypophysis. Dienestrol, which is more active than ethynil-estradiol caused less hypophyseal enlargement than the latter. Rats injected with androgens showed a slight decrease in the weight of the hypophysis, which, however, was not always significant. The adrenals were enlarged by substances (estrogens) which protected against diabetes. Androgens diminished slightly the weight of the adrenals at the doses used. This problem has been carefully studied by Pinto (1943).

The thymus was found to be atrophied in animals which had been treated with sexual hormones; androgens had a more powerful effect than estrogens at the doses used.

The uterus was considerably enlarged in the animals treated with estrogens and also, but less, in those treated with androgens. Progestational and corticoid steroids had no effect on uterine weight in these experiments.

The liver and the kidneys showed an increase in weight after treatment with estrogens and androgens; and no change after the injection of corticoids and steroids with progestational activity.

These changes in the weight of the different organs do not, however, give any evidence of their functional activity.

Changes in the pelt of the animals were remarkable in some groups. Thus, those treated with estrogens showed a yellowish tint in the hair which began to fall 10 or 20 days after the initiation of the treatment. Animals which were injected stilbestrol or ethynil-estradiol became almost completely bald after a few months. Rats injected with androgens, specially with testosterone, had coarse hair, which was not shed, so the animals had a thick pelt.

SUMMARY

The effects of several substances (daily injected during six months) on the incidence and severity of diabetes in white rats after removal of 95 per cent of the pancreas were studied and the following results were obtained:

The incidence of diabetes decreased in castrated females treated with the following estrogens: estrone, estradiol, stilbestrol, dienestrol, ethynil-estradiol and ethynil-testosterone.

In castrated males estrone and stilbestrol decreased the incidence of diabetes. The effect of other substances was not examined.

Testosterone and methyl-testosterone increased the incidence

and severity of diabetes in castrated females. Testosterone had the same effect in castrated males.

The incidence of diabetes was not modified by treatment with equilenine, progesterone, desoxycorticosterone and 17-β-ethyl-dihydro-testosterone.

Cholesterol, although it had no estrogenic effect, also produced a slight but definite decrease in the incidence of diabetes.

In summary, the substances with estrogenic action decreased the incidence of diabetes which followed subtotal pancreatectomy in the rat, and, on the contrary, the androgenic ones increased it.

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NOTES AND COMMENTS

THE CLOSED VESSEL TECHNIC FOR TESTING THYROID ACTIVITY IN MICE[1]

A simple, yet effective method for measuring thyroidal activity based upon the survival time of mice in a closed vessel has been proposed. The hyperthyroid state decreases survival time in a closed vessel (Smith, Emmens and Parkes, 1947); (Hutcheon, 1948) due to decreased resistance to anoxia (Rydin, 1928); (Campbell, 1938); (Smith, Emmens and Parkes, 1947). In contrast, the resistance to anoxia is increased by thyroidectomy (Leblond, 1944) but thiourea both increased and decreased survival time of mice in closed vessels (Smith, 1947). The varied results obtained with thiourea may have been due to drug toxicity and invited further study of the goiterogens. Varied thyroid hormone actions are known to be antagonized by testosterone propionate and by methionine (Caridroit, 1941); (Korenchevsky and Hall, 1941); (Masson and Romanchuck, 1945); (Grunt, 1948); (Handler and Follis, 1948) and their effects on decreased survival time induced by thyroid feeding were investigated.

Swiss albino mice from our colony maintained on Rockland mouse pellets were used. The mice were fed Purina fox chow during the experimental period so that thyroglobulin (Proloid,[2] a commercial preparation of thyroid globulin containing 0.2% organic iodine to conform to U.S.P. thyroid) propylthiouracil or dl methionine could be mixed in the diet. Testosterone propionate (Perandren, Ciba) was injected subcutaneously in daily amounts of 0.1 mg. The mice were 22 to 24 day old females, weighing 8.5 to 11.5 grams at the start and were studied over a 17 day period during which time ad libitum feeding was permitted and food intake was measured daily. Food was left in the cages until survival time measurements were made. To measure survival time, ordinary one pound jars commonly used for fruit canning with a capacity of 938 to 950 ml. were used. These jars could be sealed tight with a rubber washer and metal screw lid. One mouse was placed in a jar in which one half inch of wood shavings was placed and survival time estimated from the time when the lid was securely tightened until the last inspiratory gasp was seen. These studies were carried out in a room maintained at 74 to 78° F.

The data are recorded in Table 1 where it will be seen that normal control mice survived an average of 157 minutes whereas the inclusion of 0.1 per cent thyroglobulin in the diet curtailed survival to 87 minutes. With 0.02 per cent thyroglobulin in the diet, an intermediate response was obtained and

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[1] This investigation was done under contract with the Office of Naval Research, Navy Department.

[2] Proloid was supplied by Dr. Robert Kroc of the Maltine Company, Morris Plains, New Jersey, Propylthiouracil by Dr. M. L. Crossley of American Cyanamide Company, Bound Brook, New Jersey and Testosterone Propionate (Perandren) by Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, New Jersey.

survival time averaged 135 minutes. In contrast to the effect of hyperthyroidism, the addition of 0.01 per cent and 0.05 per cent of propylthiouracil to the diet did not alter normal survival time nor did the goiterogen oppose the usual effect of hyperthyroidism. The amount of propylthiouracil used was definitely goiterogenic but was not toxic in that food intake and body weight gains were normal. Thyroid weights were increased from 4.5 mg. to 9.3 and 10.8 mg. by propylthiouracil. Histological sections confirmed the goiterogenic action.

Leblond (1944) demonstrated that surgical removal of the thyroids increased resistance to anoxia whereas our results with thiouracil failed to show this effect. Since test conditions were not identical it can only be suggested that thiouracil and thyroidectomy may act differently in this regard. Nevertheless, hypothyroidism induced by thyroidectomy and by thiouracil are not entirely similar as liver weight and protein content are increased by thiouracil but not by thyroidectomy (Leathem and Seeley, 1948).

TABLE 1. INFLUENCE OF THYROGLOBULIN, PROPYLTHIOURACIL, dl METHIONINE AND TESTOSTERONE PROPIONATE ON SURVIVAL TIME OF MICE IN CLOSED VESSELS

No. of mice	Treatment	Total food intake	Body weight		Average survival time
			Start	End	
		gm.	gm.	gm.	minutes
15	Control	104.6	10.1	18.2	157 ±6.7*
9	0.1% Thyroglobulin†	114.6	9.9	19.0	87 ±4.0
10	0.02% Thyroglobulin	81.8	10.4	18.3	135 ±8.5
11	0.01% Propylthiouracil	116.3	10.4	18.3	158 ±8.2
9	0.05% Propylthiouracil	116.7	9.9	17.3	155 ±8.0
10	0.1% Thyroglobulin plus 0.05% Propylthiouracil	91.8	10.3	18.3	92 ±3.0
12	0.1% Thyroglobulin+2% Methionine	90.5	10.3	18.4	65 ±3.9
10	5% Methionine	94.9	11.0	17.3	130 ±5.9
10	0.1% Thyroglobulin plus 5% Methionine	92.5	10.4	14.2	85 ±7.2
10	0.1% Thyroglobulin plus 1.7 mg. testosterone proprionate	72.4	8.1	19.1	87.4±3.9

* $e = \sqrt{\frac{d^2}{n(n-1)}}$.

† Proloid, a commercial thyroid globulin preparation was used.

Under the conditions of our experiment, testosterone propionate and dl methionine did not antagonize the effect of hyperthyroidism on survival time but it was interesting to note that 2 per cent methionine and not 5 per cent methionine actually augmented the effect of thyroglobulin. Addition of 5 per cent methionine alone to the stock diet significantly reduced survival time of the mice but it was noted that body weight gain was subnormal despite normal food consumption. No explanation for the methionine effects are evident.

SUMMARY

The survival time of mice in closed vessels is reduced by oral administration of thyroid globulin but is unaffected by propylthiouracil. The hyperthyroid action is not counteracted by concomitant administration of propylthiouracil, testosterone propionate or dl methionine. However, 2 per cent

methionine augmented the thyroid globulin effect and 5 per cent methionine alone significantly decreased the resistance to anoxia.

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THE EFFECT OF HISTAMINE UPON THE TOLERANCE OF THE EVISCERATE RAT FOR GLUCOSE AS RELATED TO THE ADRENAL GLANDS

The administration of epinephrine to the eviscerate rat causes a decrease in glucose tolerance when insulin is present (Ingle and Nezamis, 1949). It was shown in the present study that histamine has a similar although less striking effect upon the glucose tolerance of the non-adrenalectomized, eviscerate rat and that the response does not occur in the absence of the adrenal glands. These results can be explained by the assumption that the administration of histamine causes a discharge of epinephrine from the adrenal medulla sufficient in amount to affect glucose tolerance. Ingle and Nezamis (in press) have reported indirect evidence that the increased secretion of epinephrine during the stress of muscle work has an extra-hepatic effect upon carbohydrate metabolism.

METHODS

Male rats of the Sprague-Dawley strain were fed Archer Dog Pellets. At a weight of 185 to 205 gm., the inferior vena cava was ligated between the liver and the kidneys in order to cause the development of a collateral circulation. Asepsis was preserved in this operation. When the animals reached a weight of 250 ± 2 gm., they were anesthetized with cyclopal and eviscerated by the method of Ingle (1949).

Solutions containing 0.9 per cent sodium chloride and varying concentrations of glucose (C. P. Merck) with and without regular insulin (Lilly) and with and without histamine dihydrochloride (Eastman) were infused into the saphenous vein of the right hind leg by means of a constant injection machine which delivered fluid from each of 6 syringes at the rate of 20 cc. in 24 hours. The glucose load is expressed as milligrams of glucose per 100 grams

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of rat per hour (mg/100/h). The infusions covered periods of 2 hours only. Temperature was constant at 26.5° C. Analyses of glucose were made on jugular vein blood taken at the end of the infusion period by the method of Miller and Van Slyke (1936).

EXPERIMENTS AND RESULTS

In experiment 1 (Table 1), 5 groups of eviscerate rats representing 12 pairs of rats per group were given a glucose load of 64/100/h. with insulin. The animals were grouped according to the concentration of histamine administered to one rat of each pair. Histamine concentrations of 1:100,000 and 1:50,000 failed to affect glucose tolerance. A concentration of 1:10,000 caused a small rise in blood glucose and concentrations of 1:2500 and 1:1000 caused definite rises in blood glucose.

TABLE 1. LEVELS OF BLOOD GLUCOSE IN EVISCERATE RATS AT THE END OF 2 HOURS OF INTRAVENOUS INFUSION*

Adrenals	Histamine concentration	Glucose load mgm./100/h.	Insulin units 24 hrs.	No. pairs rats	Average mgms. per cent glucose	
					Histamine	Control
Intact	1,100,000	64	4	12	116	122
Intact	1: 50,000	64	4	12	104	111
Intact	1: 10,000	64	4	12	139	116
Intact	1: 2,500	64	4	12	163	103
Intact	1: 1,000	64	4	12	180	110
Intact	1: 2,500	16	0	11	133	127
Intact	1: 1,000	16	0	12	127	116
Out	1: 2,500	64	4	14	96	108
Out	1: 1,000	64	4	12	109	97

* Each rat received the solution at the rate of 20 cc. per 24 hours.

In experiment 2 (Table 1), 2 groups of eviscerate rats were given a glucose load of 16/100/h. without insulin. The addition of histamine in a concentration of 1:2500 to one rat of each of 11 pairs and of 1:1000 to one rat of each of 12 pairs failed to affect significantly the level of blood glucose.

In experiment 3 (Table 1), 2 groups of adrenalectomized-eviscerate rats were given a glucose load of 64/100/h. with insulin. The addition of histamine in a concentration of 1:2500 to one rat of each of 14 pairs and of 1:1000 histamine to one rat of each of 12 pairs failed to affect significantly the level of blood glucose.

In experiment 4 (Table 2), all of the rats were given a glucose load of 64/100/h. with insulin. One pair of eviscerate rats and one pair of adrenalectomized-eviscerate rats were represented in each group. Histamine was given in a concentration of 1:2500 to one rat of each pair in 26 experimental groups. Histamine did not modify glucose tolerance in the adrenalectomized-eviscerate rats, but it did cause a marked rise in the non-adrenalectomized-eviscerate rats. The average level of blood glucose at the end of 2 hours in the control series was 110 ± 4.4 as compared to 170 ± 8.0 for the histamine series. This difference (60 ± 9.2) between averages is statistically reliable. The experiment was repeated using a histamine concentration of 1:1000 in 25 groups of rats under otherwise identical conditions. There was no demon-

TABLE 2. LEVELS OF BLOOD GLUCOSE IN EVISCERATE RATS AT THE END OF 2 HOURS OF INTRAVENOUS INFUSION*

Averages and Standard Deviations of Averages

Histamine concentration	No. groups rats	Blood glucose mgms. per cent			
		Eviscerate		Eviscerate-Adrenalectomized	
		Histamine	Control	Histamine	Control
1:2500	26	170 ± 8.03	110 ± 4.4	102 ± 4.47	108 ± 5.27
1:1000	25	172 ± 4.3	111 ± 4.5	111 ± 3.22	107 ± 4.72

* Each rat received a glucose load of 64/100/h. with insulin in solution at the rate of 20 cc. per 24 hours.

strable effect of histamine in the absence of the adrenal glands but in non-adrenalectomized, eviscerate rats it caused a marked rise in blood glucose. The average level of blood glucose at the end of 2 hours in the control group was 111 ± 4.5 as compared to 172 ± 4.3 for the histamine group. The difference (61 ± 6.2) is statistically reliable.

DISCUSSION

The extra-hepatic effect of histamine upon glucose tolerance under the conditions of these experiments may be mediated by the release of epinephrine from the adrenal medulla. The response of histamine in the non-adrenalectomized, eviscerate rat is similar to the response to infusions of epinephrine although less in extent (Ingle and Nezamis, 1949). During a 2-hour period of infusion both drugs cause a decrease in glucose tolerance when insulin is present but not in its absence. Epinephrine causes a decrease in glucose tolerance in the adrenalectomized-eviscerate rat (Ingle and Nezamis, in press), but the response to histamine was abolished by adrenalectomy. The injection of histamine is an adequate stimulus for the release of epinephrine by the adrenal medulla in the rat (Ingle, 1938).

These data add some indirect support to the evidence already presented (Ingle and Nezamis, in press) that the discharge of epinephrine from the adrenal medulla during stress may have an extra-hepatic effect upon glucose tolerance. Proof for the hypothesis is lacking.

The mechanism whereby epinephrine and histamine affect glucose tolerance in the eviscerate rat has not been determined. There may be inhibition of glucose utilization for energy purposes, inhibition of its conversion to glycogen or fat, and there may be an increased breakdown of tissue glycogen sparing the utilization of exogenous glucose or contributing endogenous glucose to the blood.

SUMMARY

Eviscerate male rats of 250 gms. were infused intravenously for a period of 2 hours. When a glucose load of 64/100/h. with insulin was used, the addition of histamine in concentrations of 1:2500 and 1:1000 caused significant rises in glucose tolerance. Smaller amounts of histamine had little or no effect. When a glucose load of 16/100/h. without insulin was given, the addition of histamine in concentrations of 1:2500 and 1:1000 had little or no effect upon glucose tolerance. Adrenalectomized-eviscerate rats were given a

glucose load of 64/100/h. with insulin. The addition of histamine in concentrations of 1:2500 and 1:1000 failed to affect glucose tolerance in the absence of the adrenal glands. It was concluded that the extra-hepatic effect of histamine upon glucose tolerance may be mediated by the release of epinephrine from the adrenal medulla. The response to histamine in the non-adrenalectomized-eviscerate rat is similar to the response to epinephrine. The response to histamine is abolished by adrenalectomy.

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METABOLISM OF THE STEROID HORMONES: METABOLISM OF Δ^4-ANDROSTENEDIONE-3, 17, ANDROSTANEDIONE-3, 17, AND ANDROSTANEDIOL-3 (α), 17(α) IN A HYPOGONADAL MAN[1]

In a previous investigation the in vivo conversion of Δ-androstenedione-3, 17, androstanedione-3, 17, and androstanediol-3 (α), 17 (α) to androsterone was reported (Dorfman and Hamilton, 1940). However, the yields of androsterone were low, and in the case of Δ^4-androstenedione-3, 17, only androsterone was isolated, whereas it would be expected that both androsterone and etiocholanol-3(α)-one-17 should be found. Accordingly, it seemed of interest to reinvestigate the metabolic fate of the three steroids.[2]

EXPERIMENTAL

The patient was a 51-year-old hypogonadal man who was diagnosed as having the Lawrence-Moon-Biedl syndrome (Roth, 1946). A control sample of urine collected over a thirteen day period showed a 17-ketosteroid value of 3.2 mg. per day in the alpha-ketonic neutral fraction, and less than 0.05 mg. per day in the beta-ketonic neutral fraction.

The urines, both the control and experimental samples, were extracted daily after the completion of the twenty-four-hour period. For the extraction the urine was acidified with 15% by volume of concentrated hydrochloric

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[2] We are indebted to Ciba Pharmaceutical Products, Inc. for the generous supply of Δ^4-androstenedione-3, 17, androstanedione-3, 17, and androstanediol-3(α), 17(α).

acid and refluxed for ten minutes. The acidified hydrolyzed urine was cooled, 10 grams of sodium chloride was added per 100 cc. of urine, and the mixture extracted with one-quarter volume of benzene. The benzene was separated and the extraction process repeated twice more. The combined benzene extracts were evaporated to dryness, taken up in ether, and the ketonic neutral fraction prepared as previously described (Dorfman, Cook, and Hamilton, 1939). The nonketonic fraction was reserved for future isolation studies. The ketonic fractions of both the control urine and the experimental urine were subjected to further study.

ADMINISTRATION OF Δ^4-ANDROSTENEDIONE-3, 17

The Δ^4-androstenedione-3, 17 was administered intramuscularly in peanut oil over a period of six days. For five of these days the dosage was 300 mg. per day (50 mg./cc.) and on one day 600 mg. was administered. The total dosage was 2100 mg., and the urine ewas collected during the six days of hormone administration and for the two days after the treatment was discontinued.

The total 17-ketosteroids excreted were 1182 mg. The total ketonic neutral fraction was subjected to chromatographic analyses using 30 g. of aluminum oxide (Brockmann). Carbon tetrachloride and carbon tetrachloride containing varying amounts of absolute ethanol were used for elution of the steroids.

$\Delta^{2\text{and}3}$-ANDROSTENONE-17

From fraction 2 through 5, representing either carbon tetrachloride or carbon tetrachloride containing 0.1 per cent ethanol, a total of 158.5 mg. of material, after recrystallizing from 90% methanol, melting at 97–98°C.[3] and 100–101°C. was isolated. No depression in melting point was found when the material was mixed with an authentic sample of $\Delta^{2\text{and}3}$-androstenone-17 melting at 102–2°C.

ANDROSTERONE

From three eluates (0.1 per cent ethanol in carbon tetrachloride) a total of 170 mg. of material was isolated. The melting points of the respective batches were 179–180°C., and 181–182°C. No depression in melting point was found when either of the three crops of crystals were mixed with an authentic sample of androsterone (M.P. 181–182°C.). An acetate was made which melted at 159–161°C., and when mixed with an authentic sample of androsterone acetate (M.P. 161.5–162.5°C.) the mixture melted at 160–161°C.

ETIOCHOLANOL-3 (α)-ONE-17

Seven eluated (0.2 per cent and 0.3 per cent ethanol in carbon tetrachloride) yielded crystalline material. They were combined and yielded 338 mg. of material melting at 137–138°C. and 148°C. No depression in melting point was found with an authentic sample of etiocholanol-3 (α)-one-17. An acetate M.P. 92–93°C. was prepared which did not depress the melting point of an authentic sample of etiocholanol-3(α)-one-17 acetate.

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[3] All melting points were taken with the Fischer-Johns Apparatus and are uncorrected.

ADMINISTRATION OF ANDROSTANEDIONE-3, 17

Androstanedione-3, 17 was administered orally over a period of six days. The daily dose varied between 400 and 700 mg. per day and the total amount administered was 3300 mg. Urine was collected during the period of hormone administration and for the following two days. Eighty-eight and four-tenths per cent of the ketonic neutral fraction amounting to 0.86 gram (962 mg. 17-ketosteroid) was dissolved in carbon tetrachloride and adsorbed on 16 grams of aluminum oxide (Brockmann). Fourteen eluates were collected, progressing from pure carbon tetrachloride to carbon tetrachloride containing 0.5 per cent ethanol.

Δ^{2and3}-ANDROSTENONE-17

Two hundred mg. of a compound melting at 97–98°C. was isolated from fraction 2 which was obtained by eluting the column with carbon tetrachloride. The compound did not depress the melting point of an authentic sample of Δ^{2and3}-androstenone-17. An oxime was prepared which melted at 151–152°C., the melting point was 151–152°C. The corrected yield for the entire ketonic fraction was 226 mg.

ANDROSTERONE AND ISOANDROSTERONE

Androsterone was isolated from two fractions (0.2 per cent ethanol in carbon tetrachloride). One crop of 2 mg., M.P. 176–181°C. and another of 10.5 mg., M.P. 177–180°C. was realized. Another eluate of 0.2 per cent ethanol in carbon tetrachloride yielded 228 mg. of a material M.P. 159–161°C. This melting point remained constant after repeated crystallizations. Rechromatographing on aluminum oxide, the material yielded a small amount of androsterone M.P. 182–183°C. but the bulk of the material was not purified further. The fractions from the rechromatogram were recombined and separated with digitonin in 90 per cent ethanol. The final concentration of digitonin was one per cent. The digitonin nonprecipitable fraction after recrystallization yielded crystalline material M.P. 182–184°C., which when mixed with an authentic sample of androsterone (M.P. 182–183°C.) melted at 181–183°C. The yield was 69 mg. of androsterone which amounted to 78 mg. equivalent for the entire ketonic fraction.

The digitonin precipitable fraction yielded crystalline material which had a constant melting point at 172–174°C. When mixed with an authentic sample of isoandrosterone (M.P. 172–174°C.), the melting point was 172–174°C. An acetate melted at 113.5–114.5°C. The melting point of a mixture of this acetate was not depressed when mixed with a sample of isoandrosterone acetate. The yield on the basis of the original ketonic fraction was 42 mg.

ADMINISTRATION OF ANDROSTANEDIOL-3(α), 17(α)

A total of 1900 mg. of androstanediol-3(α), 17(α) was administered over a three day period in doses of 600, 600, and 700 mg. respectively. Urine was collected during the treatment period and for the following two days. The neutral ketonic fraction, weight 0.9 gram, was dissolved in 20 cc. of carbon tetrachloride and absorbed on 18 grams of aluminum oxide. The column was eluted with fifteen 100 cc. portions of carbon tetrachloride and carbon tetrachloride containing between 0.1 and 0.5 per cent ethanol.

Δ^{2and3}-ANDROSTENONE-17

From the second carbon tetrachloride eluate, 198 mg. of crystalline material was obtained which melted at 96–98°C. after recrystallizing from aqueous methanol. No depression in melting point was observed when mixed with a sample of Δ^{2and3}-androstenone-17. An oxime was prepared which melted at 151–152°C. as compared to an authentic sample of Δ^{2and3}-androsterone-17 oxime which melted at 152–153°C. The mixture of the two melted at 151–153°C.

ANDROSTERONE

Androsterone was obtained from three fractions which were eluted with 0.2 per cent ethanol in carbon tetrachloride. One hundred and ninety-two mg. were isolated from the three fractions which had a melting point of 178–181°C. When mixed with androsterone, M.P. 180–181°C., the melting point was 180–181°C. An acetate of the isolated material melted at 159–161°C., and when mixed with an authentic sample of androsterone acetate, M.P. 161.6–162.5°C., the mixture melted at 160–162°C.

ISOANDROSTERONE

Three late fractions, which were eluted with 0.2 per cent ethanol in carbon tetrachloride, were combined and separated with digitonin. The digitonin precipitable fraction yielded 8 mg. of compound melting at 170–172°, which when mixed with isoandrosterone, M.P. 172–173°C., melted at 172–173°C. An acetate melted at 103–104°C. This was mixed with the higher melting form of isoandrosterone acetate, 114–115°C. The melting point of the mixture was 113–114°C.

DISCUSSION

Since $\Delta^{2\text{ and }3}$-androstenone-17 has been shown to be a dehydration product of androsterone which arises during the hydrolysis and extraction procedure, it is reasonable to assume that this dehydrated product, in effect, represents androsterone. Isoandrosterone may also be dehydrated during the processing of the urine to $\Delta^{2\text{ and }3}$-androstenone-17. In one experiment (Δ^4-androstenedione-3, 17) isoandrosterone was not detected, while the amounts isolated in the other two experiments were small. Therefore, the amount of $\Delta^{2\text{ and }3}$-androstenone-17 which may represent isoandrosterone is of a low order.

Approximately 32 per cent of the administered Δ^4-androstenedione-3, 17 was accounted for as androsterone and etiocholanol-3(α)-one-17, in equal proportions. Over 20 per cent of the androstanediol-3(α), 17 (α) was isolated, but the yield after androstanedione-3, 17 was only about 10 per cent of the administered material (Table 1). The low yield in the case of androstanedione 3, 17 was in part due to the isolation procedure.

TABLE 1. THE METABOLISM OF Δ^4-ANDROSTENEDIONE-3, 17, ANDROSTANEDIONE-3, 17 AND ANDROSTANEDIOL-3(α) 17(α)

Compound administered	Route of administration	Amount, mg.	Days of urine collection	Urinary 17-ketosteroids isolated in %			
				$\Delta^{2,3}$ Androstenone-17	Androsterone	Etiocholanol-3(α)-one-17	Isoandrosterone
Δ^4-Androstenedione-3,17	I.M.	2100	8	7.6	8.1	16.1	
Androstanedione-3,17	Oral	3300	8	6.8	2.4		1.3
Androstanediol-3(α), 17(α)	Oral	1900	5	10.4	10.1		0.4

SUMMARY

$\Delta^{2 \text{ and } 3}$-androstenone-17 (7.6 per cent), androsterone (8.1 per cent), and etiocholanol-3(α)-one-17 (16.1 per cent) were isolated from the urine of a hypogonadal man who received Δ^4-androstenedione-3, 17 by intramuscular injection. Considering $\Delta^{2 \text{ and } 3}$-androstenone-17 as an artifact of androsterone, over 30 per cent of the administered steroid was isolated as androsterone and etiocholanol-3(α)-one-17, in a ratio of one to one.

$\Delta^{2 \text{ and } 3}$-androstenone-17, androsterone, and isoandrosterone were isolated from the urine of the same patient after the oral administration of both androstanedione-3, 17 and androstaneidol-3(α), 17(α).

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NEW BOOK

NEUROLOGY. 4th Edition. 1949. By Roy R. Grinker, M.D. and Paul C. Bucy, M.D. Published by Charles C Thomas, Springfield, Ill., 1949. ix+1138 pp. Price $12.50.

This well-known textbook of clinical neurology has been revised by the senior author with the collaboration of Dr. Bucy. Thus to the original biologically oriented presentation of the human nervous system in health and disease are happily wedded the more recent findings of basic neurophysiology.

Much of the rather miscellaneous material of the first four chapters of the third edition has been wisely omitted in this revision, and the rest incorporated into appropriate parts of the succeeding text, giving a more even arrangement of subject matter.

The section on electroencephalography has been rewritten with the assistance of Dr. F. A. Gibbs and is modern and conservative in its treatment.

There can be only a few criticisms of this work. There are occasional misstatements, such as that on page 161 that the vegetative nervous system cannot be classified into cholinergic and adrenergic fibers. On page 208 it is stated that exophthalmos in hyperthyroidism is an effect of excessive sympathetic stimulation of the orbital muscles, an opinion with which few would agree. The discussion of the possible mechanisms of referred pain seems inadequate.

Most statements are well documented, and there is a voluminous bibliography. The book is well indexed. Generally speaking, this text can be highly recommended to any reader seeking an introduction to the field of neurology.

PAUL O. CHATFIELD

ASSOCIATION NOTICE
1950 AWARDS

THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

Fellowship Awards

The Ayerst, McKenna and Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin and in 1948 to Dr. Ernest M. Brown, Jr. Dr. Brown was re-elected for the Fellowship in 1949.

The Schering Fellowship in Endocrinology was established in 1949 and the recipient was Dr. D. Lawrence Wilson.

Association Fellowships are designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $2,500 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study. He must indicate one or more institutions where the proposed program will be followed. He must submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

The E. R. Squibb and Sons Award

The E. R. Squibb & Sons Award of $1,000 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright; and 1949—Dr. Herbert M. Evans. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocri-

nology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; and 1949—Dr. George Sayers. The work cited may be either in the field of pre-clinical or clinical endocrinology. The Award is for $1,200. If within 24 months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.

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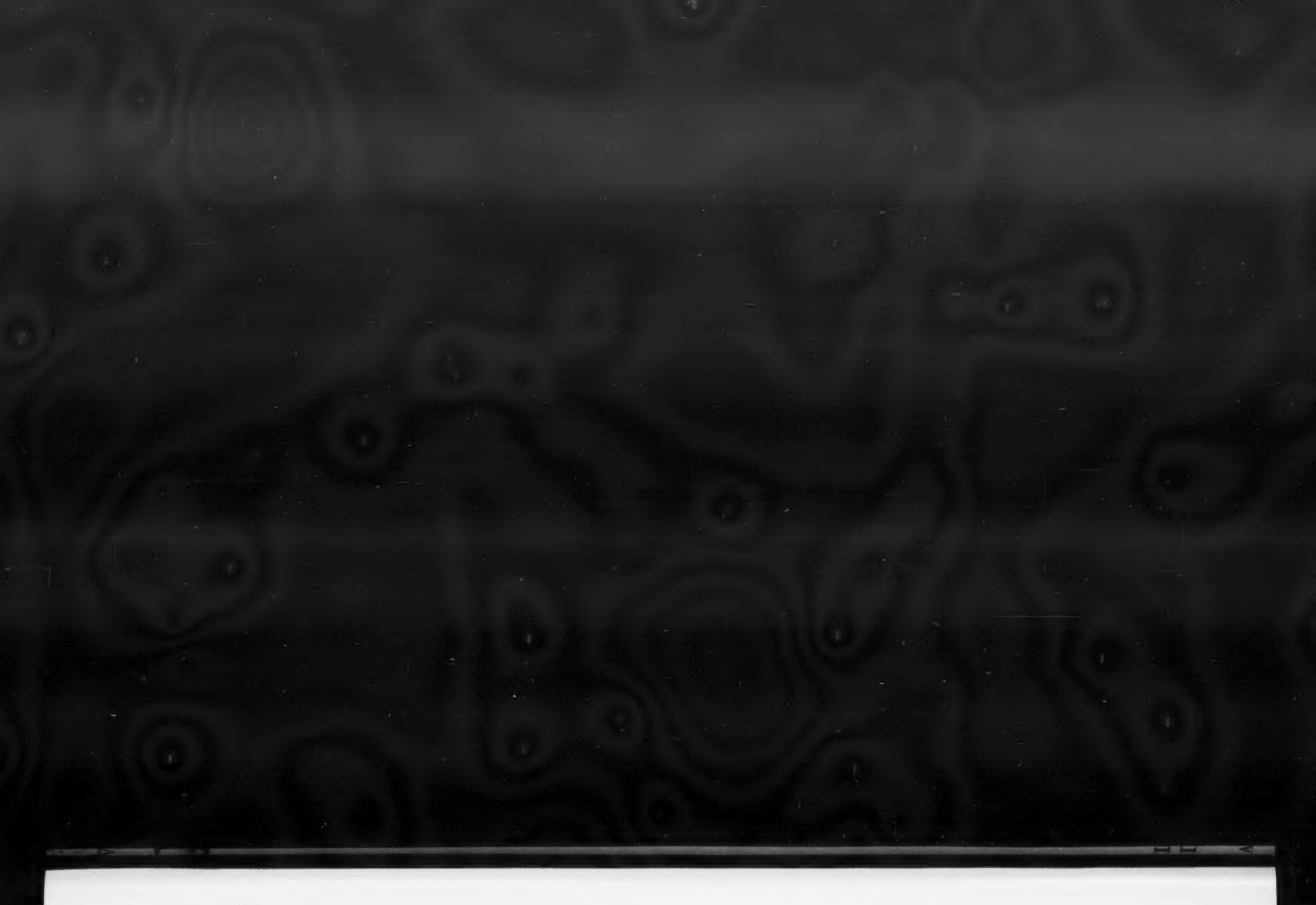
Each member has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, and reprints where possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1950.